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(54) Title: CANCER ASSOCIATED PROTEIN KINASES AND THEIR USES

(57) Abstract: Detection of expression of the provided protein kinase in cancers is useful as a diagnostic, for determining the effectiveness of drugs, and determining patient prognosis. The encoded polypeptides further provides a target for screening pharmaceutical agents effective in inhibiting the growth or metastasis of tumor cells.



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CANCER ASSOCIATED PROTEIN KINASES AND THEIR USES

INTRODUCTION

An accumulation of genetic changes underlies the development and progression of cancer, resulting in cells that differ from normal cells in their behavior, biochemistry, genetics, and microscopic appearance. Mutations in DNA that cause changes in the expression level of key proteins, or in the biological activity of proteins, are thought to be at the heart of cancer. For example, cancer can be triggered in part when genes that play a critical role in the regulation of cell division undergo mutations that lead to their over-expression. "Oncogenes" are involved in the dysregulation of growth that occurs in cancers.

Oncogene activity may involve protein kinases, enzymes that help regulate many cellular activities, particularly signaling from the cell membrane to the nucleus to initiate the cell's entrance into the cell cycle and to control other functions.

Oncogenes may be tumor susceptibility genes, which are typically up-regulated in tumor cells, or may be tumor suppressor genes, which are down-regulated or absent in tumor cells. Malignancies can arise when a tumor suppressor is lost and/or an oncogene is inappropriately activated. When such mutations occur in somatic cells, they result in the growth of sporadic tumors.

Hundreds of genes have been implicated in cancer, but in most cases relationships between these genes and their effects are poorly understood. Using massively parallel gene expression analysis, scientists can now begin to connect these genes into related pathways.

Phosphorylation is important in signal transduction mediated by receptors via extracellular biological signals such as growth factors or hormones. For example, many oncogenes are protein kinases, i.e. enzymes that catalyze protein phosphorylation reactions or are specifically regulated by phosphorylation. In addition, a kinase can have its activity regulated by one or more distinct protein kinases, resulting in specific signaling cascades.

Cloning procedures aided by homology searches of EST databases have accelerated the pace of discovery of new genes, but EST database searching remains an involved and onerous task. More than 1.6 million human EST sequences have been deposited in public databases, making it difficult to identify ESTs that represent new genes. Compounding the problems of scale are difficulties in detection associated with a high sequencing error rate and low sequence similarity between distant homologues.

Despite a long-felt need to understand and discover methods for regulating cells involved in various disease states, the complexity of signal transduction pathways has been a barrier to the development of products and processes for such regulation. Accordingly, there is a need in the art for improved methods for detecting and modulating the activity of such genes, and for treating diseases associated with the cancer and signal transduction pathway.

Relevant Literature

The use of genomic sequence in data mining for signaling proteins is discussed in Schultz et al. (2000) Nature Genetics 25:201. The MAPK protein family has been reviewed, for example by

Meskiene I, and Hirt, H. (2000) Plant Mol Biol 42(6):791-806. MAP3K has been discussed, for example, by Ing, Y.L. *et al.* (1994) Oncogene 9: 1745-1750 and also by Courseaux, A. *et al.* (1996) Genomics, 37:354-365 Serine/threonine protein kinases have been reviewed, for example, by Cross TG, *et al.* (2000) Exp Cell Res. Apr 10;256(1):34-41.

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SUMMARY OF THE INVENTION

The genetic sequences provided herein as SEQ ID NOS:1, 3, 5, 7, 9, 11 and 13 encode protein kinases that are herein shown to be over-expressed in cancer cells. Detection of expression in cancer cells is useful as a diagnostic; for determining the effectiveness and mechanism of action of therapeutic drug candidates, and for determining patient prognosis. These sequences further provides a target for screening pharmaceutical agents effective in inhibiting the growth or metastasis of tumor cells. In one embodiment of the invention, a complete nucleotide sequence of the human cDNA corresponding to the cancer associated protein kinase is provided.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph depicting the proliferation of Cos7 cells that were transfected with increasing concentrations of CaMK-X1 or vector plasmids in the presence of KCl.

Figure 2 is a graph depicting phosphorylation of CREBtide and Syntide 2 *in vitro* by CamKX1.

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Figure 3 is a graph depicting activity of transcription factors in the presence of SGK2. AP1 and NF- κ B activity was measured in HEK293 cells and in HEK293 cells stably transfected with SGK2.

Figure 4 is a graph depicting the activation of SGK2 (K 25 plasmid) by PDK1.

Figure 5 depicts the sequences of several DMPK isoforms.

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DESCRIPTION OF THE SPECIFIC EMBODIMENTS

SEQ ID NOS:1, 3, 5, 7, 9, 11 and 13 encode protein kinases that are shown to be over-expressed in cancer cells. The encoded cancer associated protein kinases of the invention provide targets for drug screening or altering expression levels, and for determining other molecular targets in kinase signal transduction pathways involved in transformation and growth of tumor cells. Detection of over-expression in cancers provides a useful diagnostic for predicting patient prognosis and probability of drug effectiveness.

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PROTEIN KINASES

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Mitogen Activated Protein Kinases. The human gene sequence encoding MAP3K11, is provided as SEQ ID NO:1, and the encoded polypeptide product is provided as SEQ ID NO: 2. Dot blot analysis of probes prepared from mRNA of tumors showed that expression of MAP3K11 is consistently up-regulated in clinical samples of human tumors.

Many of the transduction pathways in mammalian cells that involve the sequential activation of a series of signaling proteins linking the cell surface with nuclear targets are mediated by mitogen-

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activated protein kinases (MAPKs) (also called extracellular signal-regulated kinases or ERKs). In mammalian cells, three parallel MAPK pathways have been described. Generally, MAPKs are rapidly activated in response to ligand binding by both growth factor receptors that are tyrosine kinases (such as the EGF receptor) and receptors that are coupled to G proteins. Phosphorylation of tyrosine residues leads to generation of docking sites for SH2 (Src homology 2) and PTB (phosphotyrosine binding) domains of adaptor proteins. (see Lemmon *et al.* (1994) Trends Biochem Sci 19:459-63; and Pawson *et al.* (1997) Science 278:2075-80.

Mitogen-activated protein (MAP) kinases include extracellular signal-regulated protein kinase (ERK), c-Jun amino-terminal kinase (JNK), and p38 subgroups. These MAP kinase isoforms are activated by dual phosphorylation on threonine and tyrosine (Derijard *et al.* (1995) Science 267(5198):682-5). MAP3K11 is an isoform that has been described by Ing *et al.* (1994) Oncogene 9:1745-1750. It has been mapped via fluorescence in situ hybridization to 11q13.1-q13.3 (Courseaux *et al.* (1996) Genomics 37:354-365). MAP3K also shares homology, including an unusual leucine zipper-basic motif, with a family of protein kinases known as mixed lineage protein kinases.

Ing *et al.* (*supra.*) found that MAP3K contains an SH3 domain and has a long carboxy-terminal tail that exhibits proline rich motifs similar to known SH3 binding sites. SH3 domains play the role of a protein switch, which is turned on by a number of receptor-mediated signals to which it responds by changes in kinase activity and by changes in intracellular localization. It acts as part of an adapter molecule and recruits downstream proteins in a signaling pathway.

Calmodulin Kinase. The human gene sequence encoding CaMK-X1, which maps to chromosome 1q32.1-32.3, is provided as SEQ ID NO:3, and the encoded polypeptide product is provided as SEQ ID NO: 4. The open reading frame of the sequence is indicated in the seqlist of SEQ ID NO:3, and starts at position 70. Dot blot analysis of probes prepared from mRNA of tumors showed that expression of CaMK-X1 is consistently up-regulated in human tumor tissue.

Many of the intracellular physiological activities in mammalian cells that involve Ca^{++} as a second messenger are mediated by calmodulin (CaM). This ubiquitous Ca^{++} -binding protein has an ability to activate a variety of enzymes in a Ca^{++} -dependent manner. Among these enzymes are Ca^{++} and calmodulin-dependent cyclic-nucleotide phosphodiesterase (CaM-PDE) and the calmodulin-dependent kinases. Many of the CaM-kinases are activated by phosphorylation in addition to binding to CaM. The kinase may autophosphorylate, or be phosphorylated by another kinase as part of a "kinase cascade".

Each member of the CaM-kinase cascade has a catalytic domain adjacent to a regulatory region that contains an overlapping auto-inhibitory domain (AID) and the CaM-binding domain (CBD). An interaction between the AID and the catalytic domain maintains the kinase in an inactive conformation by preventing binding of protein substrate as well as Mg^{++} -ATP. Binding of Ca^{++} -CaM to the CBD alters the conformation of the overlapping AID such that it no longer interferes with substrate binding; the kinase is therefore active. As in the cases of other protein kinases, CaMKI has a catalytic cleft between its upper and lower lobes, which are responsible for binding Mg^{++} -ATP and

protein substrates, respectively. At the base of their catalytic clefts, many protein kinases, including CaMKI and CaMKIV, have an activation loop containing a threonine residue whose phosphorylation strongly augments kinase activity.

5 *Serum and Glucocorticoid-induced Protein Kinases (SGK).* The human gene sequence encoding SGK2- α is provided as SEQ ID NO:5, and the encoded polypeptide product is provided as SEQ ID NO:6. Dot blot analysis of probes prepared from mRNA of tumors showed that expression of SGK2- α is consistently up-regulated in human tumor tissue.

10 SGKs actively shuttle between the nucleus and the cytoplasm in synchrony with the cell cycle. SGK was originally identified as a glucocorticoid and osmotic stress-responsive gene; two related isoforms have been termed SGK2 and SGK3. In addition, there are two splice variants of SGK2; specifically, SGK2 α and SGK2 β . SGK2 α encodes a protein of 367 residues with a calculated molecular mass of 41.1 kDa. Although SGK 1, 2, and 3 share a high degree of sequence similarity, the mechanisms that regulate the level and activity of SGK2 and SGK3 differ significantly from those
15 that regulate SGK1. SGK2 has a peptide specificity similar to that of protein kinase B, preferentially phosphorylating Ser and Thr residues that lie in Arg-Xaa-Arg-Xaa-Xaa-Ser/Thr motifs.

 The data provided herein demonstrate that SGK2 α is activated by protein dependent kinase 1. CDK1 is a catalytic subunit of a protein kinase complex, called the M-phase promoting factor, that induces entry into mitosis and is universal among eukaryotes. Lee *et al.* (1988) Nature 333: 676-679
20 describe the regulated expression and phosphorylation of CDK1 in human and murine *in vitro* systems. Serum stimulation of human and mouse fibroblasts results in a marked increase in CDK1 transcription. Both the yeast and mammalian systems are regulated by phosphorylation of the gene product. In HeLa cells, CDK1 is the most abundant phosphotyrosine-containing protein and its phosphotyrosine content is subject to cell-cycle regulation (Draetta *et al.* (1988) Nature 336: 738-
25 744). One site of CDK1 tyrosine phosphorylation *in vivo* is selectively phosphorylated *in vitro* by a product of the SRC gene. Taxol activates CDK1 kinase in MDA-MB-435 breast cancer cells, leading to cell cycle arrest at the G2/M phase and, subsequently, apoptosis. Chemical inhibitors of CDK1 block taxol-induced apoptosis in these cells (Yu *et al.* (1998) Molec. Cell 2:581-591). Interference in this pathway is of interest in the development of therapeutic agents that affect cell cycle arrest and
30 apoptosis.

G Protein coupled Receptor Kinase. The human gene sequence encoding GRK5 is provided as SEQ ID NO:7, and the encoded polypeptide product is provided as SEQ ID NO:8. Dot blot analysis of probes prepared from mRNA of tumors showed that expression of GRK5 is consistently
35 up-regulated in clinical samples of human tumors.

 GRKs are a family of serine/threonine kinases that induce receptor desensitization by the phosphorylation of agonist-occupied or -activated receptors. GRKs transduce the binding of extracellular ligands into intracellular signaling events. To date, seven members of the GRK family have been identified. Common features of these kinases include a centrally localized catalytic

domain of approximately 240 amino acids, which shares significant sequence identity between family members, an N-terminal domain of 161-197 amino acids, and a variable length C-terminal domain. All of the GRKs can directly interact with phospholipids either via covalent modifications such as farnesylation, palmitoylation, or via lipid binding domains such as the pleckstrin homology domain, or
5 a polybasic domain.

GRK5 is a protein of approximately 67.7 kDa (see Kunapali and Benovic (1993) P.N.A.S. 90:5588-5592) and was identified by its homology with other members of the GRK family. It is expressed in a number of different tissues, including heart, placenta and lung. Autophosphorylation of GRK5 appears to activate the kinase (Pronin and Benovic (1997) P.N.A.S. 272:3806-3812).
10 GRK5 is also phosphorylated by PKC, where the major sites of PKC phosphorylation are localized within the C-terminal 26 amino acids. PKC phosphorylation significantly inhibits GRK5 activity.

Myotonic dystrophy protein kinase. The human gene sequence encoding DM-PK, is provided as SEQ ID NO:9, and the encoded polypeptide product is provided as SEQ ID NO: 10. The
15 sequence of additional isoforms is provided as SEQ ID NO:38 and SEQ ID NO:39. Dot blot analysis of probes prepared from mRNA of tumors showed that expression of DM-PK is consistently up-regulated in clinical samples of human tumors.

Human myotonic dystrophy protein kinase (DM-PK) is a member of a class of multidomain protein kinases that regulate cell size and shape in a variety of organisms (see Brook *et al.* (1992) Cell 68:799-808; and Fu *et al.* (1992) Science 255:1256-1258). DM-PK exhibits a novel catalytic activity similar to, but distinct from, related protein kinases such as protein kinase C and A, and the Rho kinases. Little is currently known about the general properties of DM-PK including domain function, substrate specificity, and potential mechanisms of regulation. Two forms of the kinase are expressed in muscle, where the larger form (the primary translation product) is proteolytically cleaved
20 near the carboxy terminus to generate the smaller. Inhibitory activity of the full-length kinase has been mapped to a pseudosubstrate autoinhibitory domain at the extreme carboxy terminus of DM-PK (see Bush *et al.* (2000) Biochemistry 39:8480-90).

Shaw *et al.* (1993) Genomics 18:673-9 demonstrated that the DM-PK gene contains 15 exons distributed over about 13 kb of genomic DNA. It encodes a protein of 624 amino acids with an
30 N-terminal domain highly homologous to cAMP-dependent serine-threonine protein kinases, an intermediate domain with a high alpha-helical content and weak similarity to various filamentous proteins, and a hydrophobic C-terminal segment. A CTG repeat is located in the 3' untranslated region of DM-PK mRNA. The unstable CTG motif is found uniquely in humans, although the flanking nucleotides are also present in mouse. The involvement of a protein kinase in myotonic dystrophy is
35 consistent with the pivotal role of such enzymes in a wide range of biochemical and cellular pathways. The autosomal dominant nature of the disease is due to a dosage deficiency.

Protein Kinase D2. The human gene sequence encoding PKD2 is provided as SEQ ID NO:11, and the encoded polypeptide product is provided as SEQ ID NO:12. Dot blot analysis of

probes prepared from mRNA of tumors showed that expression of PKD2 is consistently up-regulated in clinical samples of human tumors.

PKD2 is a human serine threonine protein kinase gene (Genbank accession number NM_016457; Sturany *et al.* (2001) *J. Biol. Chem.* 276:3310-3318). The protein sequence contains
 5 two cysteine-rich motifs at the N terminus, a pleckstrin homology domain, and a catalytic domain containing all the characteristic sequence motifs of serine protein kinases. It exhibits the strongest homology to the serine threonine protein kinases PKD/PKC μ and PKC, particularly in the duplex zinc finger-like cysteine-rich motif, in the pleckstrin homology domain and in the protein kinase domain. The mRNA of PKD2 is widely expressed in human and murine tissues. It encodes a protein with a
 10 molecular mass of 105 kDa in SDS-polyacrylamide gel electrophoresis, which is expressed in various human cell lines, including HL60 cells, which do not express PKC μ . In vivo phorbol ester binding studies demonstrated a concentration-dependent binding of [³H]phorbol 12,13-dibutyrate to PKD2. The addition of phorbol 12,13-dibutyrate in the presence of dioleoylphosphatidylserine stimulated the autophosphorylation of PKD2 in a synergistic fashion. Phorbol esters also stimulated
 15 autophosphorylation of PKD2 in intact cells. Phosphorylation of Ser876 of PKD2 correlated with the activation status of the kinase.

DIAGNOSTIC METHODS

Determination of the presence of any one of SEQ ID NOS:1, 3, 5, 7, 9, 11 and 13 is used in
 20 the diagnosis, typing and staging of tumors. Detection of the presence of the sequence is performed by the use of a specific binding pair member to quantitate the specific protein, DNA or RNA present in a patient sample. Generally the sample will be a biopsy or other cell sample from the tumor. Where the tumor has metastasized, blood samples may be analyzed.

25 SPECIFIC BINDING MEMBERS

In a typical assay, a tissue sample, *e.g.* biopsy, blood sample, *etc.* is assayed for the presence of a cancer associated kinase corresponding to SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13 specific sequences by combining the sample with a SEQ ID NOS:1, 3, 5, 7, 9, 11 and 13 specific binding member, and detecting directly or indirectly the presence of the complex formed between the two
 30 members. The term "specific binding member" as used herein refers to a member of a specific binding pair, *i.e.* two molecules where one of the molecules through chemical or physical means specifically binds to the other molecule. One of the molecules will be a nucleic acid corresponding to SEQ ID NOS:1, 3, 5, 7, 9, 11 and 13 or a polypeptide encoded by the nucleic acid, which can include any protein substantially similar to the amino acid sequence provided in SEQ ID NOS:2, 4, 6, 8, 10,
 35 12, 14, 38 or 39 or a fragment thereof; or any nucleic acid substantially similar to the nucleotide sequence provided in SEQ ID NOS:1, 3, 5, 7, 9, 11 and 13, or a fragment thereof. The complementary members of a specific binding pair are sometimes referred to as a ligand and receptor.

Binding pairs of interest include antigen and antibody specific binding pairs, peptide-MHC
 40 antigen and T cell receptor pairs; complementary nucleotide sequences (including nucleic acid

sequences used as probes and capture agents in DNA hybridization assays); kinase protein and substrate pairs; autologous monoclonal antibodies, and the like. The specific binding pairs may include analogs, derivatives and fragments of the original specific binding member. For example, an antibody directed to a protein antigen may also recognize peptide fragments, chemically synthesized peptidomimetics, labeled protein, derivatized protein, *etc.* so long as an epitope is present.

Nucleic acid sequences. In another embodiment of the invention, nucleic acids are used as a specific binding member. Sequences for detection are complementary to a one of the provided cancer associated kinase corresponding to SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13. The nucleic acids of the invention include nucleic acids having a high degree of sequence similarity or sequence identity to one of SEQ ID NOS:1, 3, 5, 7, 9, 11 and 13. Sequence identity can be determined by hybridization under stringent conditions, for example, at 50°C or higher and 0.1XSSC (9 mM saline/0.9 mM sodium citrate). Hybridization methods and conditions are well known in the art, see, *e.g.*, U.S. patent 5,707,829. Nucleic acids that are substantially identical to the provided nucleic acid sequence, *e.g.* allelic variants, genetically altered versions of the gene, *etc.*, bind to SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13 under stringent hybridization conditions.

The nucleic acids can be cDNAs or genomic DNAs, as well as fragments thereof. The term "cDNA" as used herein is intended to include all nucleic acids that share the arrangement of sequence elements found in native mature mRNA species, where sequence elements are exons and 3' and 5' non-coding regions. Normally mRNA species have contiguous exons, with the intervening introns, when present, being removed by nuclear RNA splicing, to create a continuous open reading frame encoding a polypeptide of the invention.

A genomic sequence of interest comprises the nucleic acid present between the initiation codon and the stop codon, as defined in the listed sequences, including all of the introns that are normally present in a native chromosome. It can further include the 3' and 5' untranslated regions found in the mature mRNA. It can further include specific transcriptional and translational regulatory sequences, such as promoters, enhancers, *etc.*, including about 1 kb, but possibly more, of flanking genomic DNA at either the 5' or 3' end of the transcribed region. The genomic DNA flanking the coding region, either 3' or 5', or internal regulatory sequences as sometimes found in introns, contains sequences required for proper tissue, stage-specific, or disease-state specific expression, and are useful for investigating the up-regulation of expression in tumor cells.

Probes specific to the nucleic acid of the invention can be generated using the nucleic acid sequence disclosed in SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13. The probes are preferably at least about 18 nt, 25nt, 50 nt or more of the corresponding contiguous sequence of SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13, and are usually less than about 2, 1, or 0.5 kb in length. Preferably, probes are designed based on a contiguous sequence that remains unmasked following application of a masking program for masking low complexity, *e.g.* BLASTX. Double or single stranded fragments can be obtained from the DNA sequence by chemically synthesizing oligonucleotides in accordance with conventional methods, by restriction enzyme digestion, by PCR amplification, *etc.* The probes can be labeled, for example, with a radioactive, biotinylated, or fluorescent tag.

The nucleic acids of the subject invention are isolated and obtained in substantial purity, generally as other than an intact chromosome. Usually, the nucleic acids, either as DNA or RNA, will be obtained substantially free of other naturally-occurring nucleic acid sequences, generally being at least about 50%, usually at least about 90% pure and are typically "recombinant," e.g., flanked by one or more nucleotides with which it is not normally associated on a naturally occurring chromosome.

The nucleic acids of the invention can be provided as a linear molecule or within a circular molecule, and can be provided within autonomously replicating molecules (vectors) or within molecules without replication sequences. Expression of the nucleic acids can be regulated by their own or by other regulatory sequences known in the art. The nucleic acids of the invention can be introduced into suitable host cells using a variety of techniques available in the art, such as transferrin polycation-mediated DNA transfer, transfection with naked or encapsulated nucleic acids, liposome-mediated DNA transfer, intracellular transportation of DNA-coated latex beads, protoplast fusion, viral infection, electroporation, gene gun, calcium phosphate-mediated transfection, and the like.

For use in amplification reactions, such as PCR, a pair of primers will be used. The exact composition of the primer sequences is not critical to the invention, but for most applications the primers will hybridize to the subject sequence under stringent conditions, as known in the art. It is preferable to choose a pair of primers that will generate an amplification product of at least about 50 nt, preferably at least about 100 nt. Algorithms for the selection of primer sequences are generally known, and are available in commercial software packages. Amplification primers hybridize to complementary strands of DNA, and will prime towards each other. For hybridization probes, it may be desirable to use nucleic acid analogs, in order to improve the stability and binding affinity. The term "nucleic acid" shall be understood to encompass such analogs.

Antibodies. The polypeptides of the invention may be used for the production of antibodies, where short fragments provide for antibodies specific for the particular polypeptide, and larger fragments or the entire protein allow for the production of antibodies over the surface of the polypeptide. As used herein, the term "antibodies" includes antibodies of any isotype, fragments of antibodies which retain specific binding to antigen, including, but not limited to, Fab, Fv, scFv, and Fd fragments, chimeric antibodies, humanized antibodies, single-chain antibodies, and fusion proteins comprising an antigen-binding portion of an antibody and a non-antibody protein. The antibodies may be detectably labeled, e.g., with a radioisotope, an enzyme which generates a detectable product, a green fluorescent protein, and the like. The antibodies may be further conjugated to other moieties, such as members of specific binding pairs, e.g., biotin (member of biotin-avidin specific binding pair), and the like. The antibodies may also be bound to a solid support, including, but not limited to, polystyrene plates or beads, and the like.

"Antibody specificity", in the context of antibody-antigen interactions, is a term well understood in the art, and indicates that a given antibody binds to a given antigen, wherein the binding can be inhibited by that antigen or an epitope thereof which is recognized by the antibody,

and does not substantially bind to unrelated antigens. Methods of determining specific antibody binding are well known to those skilled in the art, and can be used to determine the specificity of antibodies of the invention for a polypeptide, particularly a human polypeptide corresponding to SEQ ID NOS:2, 4, 6, 8, 10 or 12.

5 Antibodies are prepared in accordance with conventional ways, where the expressed polypeptide or protein is used as an immunogen, by itself or conjugated to known immunogenic carriers, e.g. KLH, pre-S HBsAg, other viral or eukaryotic proteins, or the like. Various adjuvants may be employed, with a series of injections, as appropriate. For monoclonal antibodies, after one or more booster injections, the spleen is isolated, the lymphocytes immortalized by cell fusion, and then
10 screened for high affinity antibody binding. The immortalized cells, *i.e.* hybridomas, producing the desired antibodies may then be expanded. For further description, see *Monoclonal Antibodies: A Laboratory Manual*, Harlow and Lane eds., Cold Spring Harbor Laboratories, Cold Spring Harbor, New York, 1988. If desired, the mRNA encoding the heavy and light chains may be isolated and mutagenized by cloning in *E. coli*, and the heavy and light chains mixed to further enhance the
15 affinity of the antibody. Alternatives to *in vivo* immunization as a method of raising antibodies include binding to phage display libraries, usually in conjunction with *in vitro* affinity maturation.

METHODS FOR QUANTITATION OF NUCLEIC ACIDS

 Nucleic acid reagents derived from the sequence of SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13 are
20 used to screen patient samples, e.g. biopsy-derived tumors, inflammatory samples such as arthritic synovium, *etc.*, for amplified DNA in the cell, or increased expression of the corresponding mRNA or protein. DNA-based reagents are also designed for evaluation of chromosomal loci implicated in certain diseases e.g. for use in loss-of-heterozygosity (LOH) studies, or design of primers based on coding sequences.

25 The polynucleotides of the invention can be used to detect differences in expression levels between two cells, e.g., as a method to identify abnormal or diseased tissue in a human. The tissue suspected of being abnormal or diseased can be derived from a different tissue type of the human, but preferably it is derived from the same tissue type; for example, an intestinal polyp or other abnormal growth should be compared with normal intestinal tissue. The normal tissue can be the
30 same tissue as that of the test sample, or any normal tissue of the patient, especially those that express the polynucleotide-related gene of interest (e.g., brain, thymus, testis, heart, prostate, placenta, spleen, small intestine, skeletal muscle, pancreas, and the mucosal lining of the colon, *etc.*). A difference between the polynucleotide-related gene, mRNA, or protein in the two tissues which are compared, for example, in molecular weight, amino acid or nucleotide sequence, or
35 relative abundance, indicates a change in the gene, or a gene which regulates it, in the tissue of the human that was suspected of being diseased.

 The subject nucleic acid and/or polypeptide compositions may be used to analyze a patient sample for the presence of polymorphisms associated with a disease state. Biochemical studies may be performed to determine whether a sequence polymorphism in a coding region or control
40 regions is associated with disease, particularly cancers and other growth abnormalities. Diseases of

interest may also include other hyperproliferative disorders. Disease associated polymorphisms may include deletion or truncation of the gene, mutations that alter expression level, that affect the binding activity of the protein, the kinase activity domain, *etc.*

Changes in the promoter or enhancer sequence that may affect expression levels of can be compared to expression levels of the normal allele by various methods known in the art. Methods for determining promoter or enhancer strength include quantitation of the expressed natural protein; insertion of the variant control element into a vector with a reporter gene such as beta-galactosidase, luciferase, chloramphenicol acetyltransferase, *etc.* that provides for convenient quantitation; and the like.

A number of methods are available for analyzing nucleic acids for the presence of a specific sequence, *e.g.* upregulated expression. Cells that express SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13 may be used as a source of mRNA, which may be assayed directly or reverse transcribed into cDNA for analysis. The nucleic acid may be amplified by conventional techniques, such as the polymerase chain reaction (PCR), to provide sufficient amounts for analysis. The use of the polymerase chain reaction is described in Saiki *et al.* (1985) Science 239:487, and a review of techniques may be found in Sambrook, *et al.* Molecular Cloning: A Laboratory Manual, CSH Press 1989, pp.14.2-14.33.

A detectable label may be included in an amplification reaction. Suitable labels include fluorochromes, *e.g.* fluorescein isothiocyanate (FITC), rhodamine, Texas Red, phycoerythrin, allophycocyanin, 6-carboxyfluorescein (6-FAM), 2,7-dimethoxy-4,5-dichloro-6-carboxyfluorescein (JOE), 6-carboxy-X-rhodamine (ROX), 6-carboxy-2,4,7,4,7-hexachlorofluorescein (HEX), 5-carboxyfluorescein (5-FAM) or N,N,N,N-tetramethyl-6-carboxyrhodamine (TAMRA), radioactive labels, *e.g.* ^{32}P , ^{35}S , ^3H ; *etc.* The label may be a two stage system, where the amplified DNA is conjugated to biotin, haptens, *etc.* having a high affinity binding partner, *e.g.* avidin, specific antibodies, *etc.*, where the binding partner is conjugated to a detectable label. The label may be conjugated to one or both of the primers. Alternatively, the pool of nucleotides used in the amplification is labeled, so as to incorporate the label into the amplification product.

The sample nucleic acid, *e.g.* amplified or cloned fragment, is analyzed by one of a number of methods known in the art. Probes may be hybridized to northern or dot blots, or liquid hybridization reactions performed. The nucleic acid may be sequenced by dideoxy or other methods, and the sequence of bases compared to a wild-type sequence. Single strand conformational polymorphism (SSCP) analysis, denaturing gradient gel electrophoresis (DGGE), and heteroduplex analysis in gel matrices are used to detect conformational changes created by DNA sequence variation as alterations in electrophoretic mobility. Fractionation is performed by gel or capillary electrophoresis, particularly acrylamide or agarose gels.

Arrays provide a high throughput technique that can assay a large number of polynucleotides in a sample. In one aspect of the invention, an array is constructed comprising one or more of SEQ ID NOS:1, 3, 5, 7, 9, 11 and 13, preferably comprising all of these sequences, which array may further comprise other sequences known to be up- or down-regulated in tumor cells. This technology can be used as a tool to test for differential expression.

A variety of methods of producing arrays, as well as variations of these methods, are known in the art and contemplated for use in the invention. For example, arrays can be created by spotting polynucleotide probes onto a substrate (e.g., glass, nitrocellulose, etc.) in a two-dimensional matrix or array having bound probes. The probes can be bound to the substrate by either covalent bonds or by non-specific interactions, such as hydrophobic interactions. Samples of nucleic acids can be detectably labeled (e.g., using radioactive or fluorescent labels) and then hybridized to the probes. Double stranded nucleic acids, comprising the labeled sample polynucleotides bound to probe nucleic acids, can be detected once the unbound portion of the sample is washed away. Alternatively, the nucleic acids of the test sample can be immobilized on the array, and the probes detectably labeled.

Techniques for constructing arrays and methods of using these arrays are described in, for example, Schena *et al.* (1996) Proc Natl Acad Sci U S A. 93(20):10614-9; Schena *et al.* (1995) Science 270(5235):467-70; Shalon *et al.* (1996) Genome Res. 6(7):639-45, USPN 5,807,522, EP 799 897; WO 97/29212; WO 97/27317; EP 785 280; WO 97/02357; USPN 5,593,839; USPN 5,578,832; EP 728 520; USPN 5,599,695; EP 721 016; USPN 5,556,752; WO 95/22058; and USPN 5,631,734.

Arrays can be used to, for example, examine differential expression of genes and can be used to determine gene function. For example, arrays can be used to detect differential expression of SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13, where expression is compared between a test cell and control cell (e.g., cancer cells and normal cells). High expression of a particular message in a cancer cell, which is not observed in a corresponding normal cell, indicates a cancer specific gene product. Exemplary uses of arrays are further described in, for example, Pappalarado *et al.* (1998) Sem. Radiation Oncol. 8:217; and Ramsay. (1998) Nature Biotechnol. 16:40. Furthermore, many variations on methods of detection using arrays are well within the skill in the art and within the scope of the present invention. For example, rather than immobilizing the probe to a solid support, the test sample can be immobilized on a solid support which is then contacted with the probe.

POLYPEPTIDE ANALYSIS

Screening for expression of the subject sequences may be based on the functional or antigenic characteristics of the protein. Protein truncation assays are useful in detecting deletions that may affect the biological activity of the protein. Various immunoassays designed to detect polymorphisms in proteins encoded by SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13 may be used in screening. Where many diverse genetic mutations lead to a particular disease phenotype, functional protein assays have proven to be effective screening tools. The activity of the encoded protein in kinase assays, etc., may be determined by comparison with the wild-type protein.

A sample is taken from a patient with cancer. Samples, as used herein, include biological fluids such as blood; organ or tissue culture derived fluids; etc. Biopsy samples or other sources of carcinoma cells are of particular interest, e.g. tumor biopsy, etc. Also included in the term are derivatives and fractions of such cells and fluids. The number of cells in a sample will generally be at least about 10^3 , usually at least 10^4 , and may be about 10^5 or more. The cells may be dissociated, in

the case of solid tissues, or tissue sections may be analyzed. Alternatively a lysate of the cells may be prepared.

Detection may utilize staining of cells or histological sections, performed in accordance with conventional methods. The antibodies or other specific binding members of interest are added to the cell sample, and incubated for a period of time sufficient to allow binding to the epitope, usually at least about 10 minutes. The antibody may be labeled with radioisotopes, enzymes, fluorescers, chemiluminescers, or other labels for direct detection. Alternatively, a second stage antibody or reagent is used to amplify the signal. Such reagents are well known in the art. For example, the primary antibody may be conjugated to biotin, with horseradish peroxidase-conjugated avidin added as a second stage reagent. Final detection uses a substrate that undergoes a color change in the presence of the peroxidase. The absence or presence of antibody binding may be determined by various methods, including flow cytometry of dissociated cells, microscopy, radiography, scintillation counting, etc.

An alternative method for diagnosis depends on the *in vitro* detection of binding between antibodies and the cancer associated kinase corresponding to SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13 in a lysate. Measuring the concentration of the target protein in a sample or fraction thereof may be accomplished by a variety of specific assays. A conventional sandwich type assay may be used. For example, a sandwich assay may first attach specific antibodies to an insoluble surface or support. The particular manner of binding is not crucial so long as it is compatible with the reagents and overall methods of the invention. They may be bound to the plates covalently or non-covalently, preferably non-covalently.

The insoluble supports may be any compositions to which polypeptides can be bound, which is readily separated from soluble material, and which is otherwise compatible with the overall method. The surface of such supports may be solid or porous and of any convenient shape. Examples of suitable insoluble supports to which the receptor is bound include beads, e.g. magnetic beads, membranes and microtiter plates. These are typically made of glass, plastic (e.g. polystyrene), polysaccharides, nylon or nitrocellulose. Microtiter plates are especially convenient because a large number of assays can be carried out simultaneously, using small amounts of reagents and samples.

Patient sample lysates are then added to separately assayable supports (for example, separate wells of a microtiter plate) containing antibodies. Preferably, a series of standards, containing known concentrations of the test protein is assayed in parallel with the samples or aliquots thereof to serve as controls. Preferably, each sample and standard will be added to multiple wells so that mean values can be obtained for each. The incubation time should be sufficient for binding, generally, from about 0.1 to 3 hr is sufficient. After incubation, the insoluble support is generally washed of non-bound components. Generally, a dilute non-ionic detergent medium at an appropriate pH, generally 7-8, is used as a wash medium. From one to six washes may be employed, with sufficient volume to thoroughly wash non-specifically bound proteins present in the sample.

After washing, a solution containing a second antibody is applied. The antibody will bind to one of the proteins encoded by SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13 with sufficient specificity such that

it can be distinguished from other components present. The second antibodies may be labeled to facilitate direct, or indirect quantification of binding. Examples of labels that permit direct measurement of second receptor binding include radiolabels, such as ^3H or ^{125}I , fluorescers, dyes, beads, chemiluminescers, colloidal particles, and the like. Examples of labels that permit indirect measurement of binding include enzymes where the substrate may provide for a colored or fluorescent product. In a preferred embodiment, the antibodies are labeled with a covalently bound enzyme capable of providing a detectable product signal after addition of suitable substrate. Examples of suitable enzymes for use in conjugates include horseradish peroxidase, alkaline phosphatase, malate dehydrogenase and the like. Where not commercially available, such antibody-enzyme conjugates are readily produced by techniques known to those skilled in the art. The incubation time should be sufficient for the labeled ligand to bind available molecules. Generally, from about 0.1 to 3 hr is sufficient, usually 1 hr sufficing.

After the second binding step, the insoluble support is again washed free of non-specifically bound material, leaving the specific complex formed between the target protein and the specific binding member. The signal produced by the bound conjugate is detected by conventional means. Where an enzyme conjugate is used, an appropriate enzyme substrate is provided so a detectable product is formed.

Other immunoassays are known in the art and may find use as diagnostics. Ouchterlony plates provide a simple determination of antibody binding. Western blots may be performed on protein gels or protein spots on filters, using a detection system specific for the cancer associated kinase corresponding to SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13 as desired, conveniently using a labeling method as described for the sandwich assay.

In some cases, a competitive assay will be used. In addition to the patient sample, a competitor to the targeted protein is added to the reaction mix. The competitor and the cancer associated kinase corresponding to SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13 compete for binding to the specific binding partner. Usually, the competitor molecule will be labeled and detected as previously described, where the amount of competitor binding will be proportional to the amount of target protein present. The concentration of competitor molecule will be from about 10 times the maximum anticipated protein concentration to about equal concentration in order to make the most sensitive and linear range of detection.

In some embodiments, the methods are adapted for use *in vivo*, e.g., to locate or identify sites where cancer cells are present. In these embodiments, a detectably-labeled moiety, e.g., an antibody, which is specific for the protein encoded by one of SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13 is administered to an individual (e.g., by injection), and labeled cells are located using standard imaging techniques, including, but not limited to, magnetic resonance imaging, computed tomography scanning, and the like. In this manner, cancer cells are differentially labeled.

The detection methods can be provided as part of a kit. Thus, the invention further provides kits for detecting the presence of an mRNA corresponding to SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13, and/or a polypeptide encoded thereby, in a biological sample. Procedures using these kits can be performed by clinical laboratories, experimental laboratories, medical practitioners, or private

individuals. The kits of the invention for detecting a polypeptide comprise a moiety that specifically binds the polypeptide, which may be a specific antibody. The kits of the invention for detecting a nucleic acid comprise a moiety that specifically hybridizes to such a nucleic acid. The kit may optionally provide additional components that are useful in the procedure, including, but not limited to, buffers, developing reagents, labels, reacting surfaces, means for detection, control samples, standards, instructions, and interpretive information.

SAMPLES FOR ANALYSIS

Sample of interest include tumor tissue, e.g. excisions, biopsies, blood samples where the tumor is metastatic, etc. Of particular interest are solid tumors, e.g. carcinomas, and include, without limitation, tumors of the liver and colon. Liver cancers of interest include hepatocellular carcinoma (primary liver cancer). Also called hepatoma, this is the most common form of primary liver cancer. Chronic infection with hepatitis B and C increases the risk of developing this type of cancer. Other causes include cancer-causing substances, alcoholism, and chronic liver cirrhosis. Other liver cancers of interest for analysis by the subject methods include hepatocellular adenoma, which are benign tumors occurring most often in women of childbearing age; hemangioma, which are a type of benign tumor comprising a mass of abnormal blood vessels, cholangiocarcinoma, which originates in the lining of the bile channels in the liver or in the bile ducts; hepatoblastoma, which is common in infants and children; angiosarcoma, which is a rare cancer that originates in the blood vessels of the liver; and bile duct carcinoma and liver cysts. Cancers originating in the lung, breast, colon, pancreas and stomach and blood cells commonly are found in the liver after they become metastatic.

Also of interest are colon cancers. Types of polyps of the colon and rectum include polyps, which are any mass of tissue that arises from the bowel wall and protrudes into the lumen. Polyps may be sessile or pedunculated and vary considerably in size. Such lesions are classified histologically as tubular adenomas, tubulovillous adenomas (villoglandular polyps), villous (papillary) adenomas (with or without adenocarcinoma), hyperplastic polyps, hamartomas, juvenile polyps, polypoid carcinomas, pseudopolyps, lipomas, leiomyomas, or other rarer tumors.

SCREENING METHODS

Target Screening. Reagents specific for SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13 are used to identify targets of the encoded protein in tumor cells. For example, one of the nucleic acid coding sequences may be introduced into a tumor cell using an inducible expression system. Suitable positive and negative controls are included. Transient transfection assays, e.g. using adenovirus vectors, may be performed. The cell system allows a comparison of the pattern of gene expression in transformed cells with or without expression of the kinase. Alternatively, phosphorylation patterns after induction of expression are examined. Gene expression of putative target genes may be monitored by Northern blot or by probing microarrays of candidate genes with the test sample and a negative control where gene expression of the kinase is not induced. Patterns of phosphorylation may be monitored by incubation of the cells or lysate with labeled phosphate, followed by 1 or 2

dimensional protein gel analysis, and identification of the targets by MALDI, micro-sequencing, western blot analysis, *etc.*, as known in the art.

5 Some of the potential target genes of the subject cancer associated kinase corresponding to SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13 identified by this method will be secondary or tertiary in a complex cascade of gene expression or signaling. To identify primary targets of the subject kinase activation, expression or phosphorylation will be examined early after induction of expression (within 1-2 hours) or after blocking later steps in the cascade with cycloheximide.

10 Target genes or proteins identified by this method may be analyzed for expression in primary patient samples as well. The data for the subject cancer associated kinase corresponding to SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13 and target gene expression may be analyzed using statistical analysis to establish a correlation.

15 *Compound Screening.* The availability of a number of components in signaling pathways allows *in vitro* reconstruction of the pathway, and/or assessment of kinase action on targets. Two or more of the components may be combined *in vitro*, and the behavior assessed in terms of activation of transcription of specific target sequences; modification of protein components, *e.g.* proteolytic processing, phosphorylation, methylation, *etc.*; ability of different protein components to bind to each other *etc.* The components may be modified by sequence deletion, substitution, *etc.* to determine the functional role of specific domains.

20 Compound screening may be performed using an *in vitro* model, a genetically altered cell or animal, or purified protein corresponding to any one of SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13. One can identify ligands or substrates that bind to, modulate or mimic the action of the encoded polypeptide. Areas of investigation include the development of treatments for hyper-proliferative disorders, *e.g.* cancer, restenosis, osteoarthritis, metastasis, *etc.*

25 The polypeptides include those encoded by SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13, as well as nucleic acids that, by virtue of the degeneracy of the genetic code, are not identical in sequence to the disclosed nucleic acids, and variants thereof. Variant polypeptides can include amino acid (aa) substitutions, additions or deletions. The amino acid substitutions can be conservative amino acid substitutions or substitutions to eliminate non-essential amino acids, such as to alter a glycosylation site, a phosphorylation site or an acetylation site, or to minimize misfolding by substitution or deletion of one or more cysteine residues that are not necessary for function. Variants can be designed so as to retain or have enhanced biological activity of a particular region of the protein (*e.g.*, a functional domain and/or, where the polypeptide is a member of a protein family, a region associated with a consensus sequence). Variants also include fragments of the polypeptides disclosed herein, 30 particularly biologically active fragments and/or fragments corresponding to functional domains. Fragments of interest will typically be at least about 10 aa to at least about 15 aa in length, usually at least about 50 aa in length, and can be as long as 300 aa in length or longer, but will usually not exceed about 500 aa in length, where the fragment will have a contiguous stretch of amino acids that is identical to a polypeptide encoded by SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13, or a homolog thereof.

Transgenic animals or cells derived therefrom are also used in compound screening. Transgenic animals may be made through homologous recombination, where the normal locus corresponding to SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13 is altered. Alternatively, a nucleic acid construct is randomly integrated into the genome. Vectors for stable integration include plasmids, retroviruses and other animal viruses, YACs, and the like. A series of small deletions and/or substitutions may be made in the coding sequence to determine the role of different exons in kinase activity, oncogenesis, signal transduction, *etc.* Of interest is the use of SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13 to construct transgenic animal models for cancer, where expression of the corresponding kinase is specifically reduced or absent. Specific constructs of interest include antisense sequences that block expression of the targeted gene and expression of dominant negative mutations. A detectable marker, such as lac Z may be introduced into the locus of interest, where up-regulation of expression will result in an easily detected change in phenotype. One may also provide for expression of the target gene or variants thereof in cells or tissues where it is not normally expressed or at abnormal times of development. By providing expression of the target protein in cells in which it is not normally produced, one can induce changes in cell behavior, *e.g.* in the control of cell growth and tumorigenesis.

Compound screening identifies agents that modulate function of the cancer associated kinase corresponding to SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13. Agents that mimic its function are predicted to activate the process of cell division and growth. Conversely, agents that inhibit function may inhibit transformation. Of particular interest are screening assays for agents that have a low toxicity for human cells. A wide variety of assays may be used for this purpose, including labeled *in vitro* protein-protein binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, and the like. Knowledge of the 3-dimensional structure of the encoded protein, derived from crystallization of purified recombinant protein, could lead to the rational design of small drugs that specifically inhibit activity. These drugs may be directed at specific domains, *e.g.* the kinase catalytic domain, the regulatory domain, the auto-inhibitory domain, *etc.*

The term "agent" as used herein describes any molecule, *e.g.* protein or pharmaceutical, with the capability of altering or mimicking the physiological function of a cancer associated kinase corresponding to SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13. Generally a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a differential response to the various concentrations. Typically one of these concentrations serves as a negative control, *i.e.* at zero concentration or below the level of detection.

Candidate agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 50 and less than about 2,500 daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate

agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of
5 randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries.
10 Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, *etc.* to produce structural analogs.

Where the screening assay is a binding assay, one or more of the molecules may be joined to a label, where the label can directly or indirectly provide a detectable signal. Various labels include radioisotopes, fluorescers, chemiluminescers, enzymes, specific binding molecules, particles,
15 *e.g.* magnetic particles, and the like. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin, *etc.* For the specific binding members, the complementary member would normally be labeled with a molecule that provides for detection, in accordance with known procedures.

A variety of other reagents may be included in the screening assay. These include reagents
20 like salts, neutral proteins, *e.g.* albumin, detergents, *etc.* that are used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Reagents that improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, *etc.* may be used. The mixture of components are added in any order that provides for the requisite binding. Incubations are performed at any suitable temperature, typically between 4 and 40° C.
25 Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high-throughput screening. Typically between 0.1 and 1 hours will be sufficient.

Other assays of interest detect agents that mimic the function of a cancer associated kinase corresponding to SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13. For example, an expression construct comprising the gene may be introduced into a cell line under conditions that allow expression. The
30 level of kinase activity is determined by a functional assay, for example detection of protein phosphorylation. Alternatively, candidate agents are added to a cell that lacks the functional cancer associated kinase corresponding to SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13, and screened for the ability to reproduce the activity in a functional assay.

The compounds having the desired pharmacological activity may be administered in a
35 physiologically acceptable carrier to a host for treatment of cancer, *etc.* The compounds may also be used to enhance function in wound healing, cell growth, *etc.* The inhibitory agents may be administered in a variety of ways, orally, topically, parenterally *e.g.* subcutaneously, intraperitoneally, by viral infection, intravascularly, *etc.* Topical treatments are of particular interest. Depending upon the manner of introduction, the compounds may be formulated in a variety of ways. The

concentration of therapeutically active compound in the formulation may vary from about 0.1-10 wt %.

Formulations. The compounds of this invention can be incorporated into a variety of formulations for therapeutic administration. Particularly, agents that modulate activity of a cancer associated kinase corresponding to SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13, or polypeptides and analogs thereof are formulated for administration to patients for the treatment of cells where the target activity is undesirably high or low, e.g. to reduce the level of activity in cancer cells. More particularly, the compounds of the present invention can be formulated into pharmaceutical compositions by combination with appropriate, pharmaceutically acceptable carriers or diluents, and may be formulated into preparations in solid, semi-solid, liquid or gaseous forms, such as tablets, capsules, powders, granules, ointments, solutions, suppositories, injections, inhalants, gels, microspheres, and aerosols. As such, administration of the compounds can be achieved in various ways, including oral, buccal, rectal, parenteral, intraperitoneal, intradermal, transdermal, intra-tracheal, etc., administration. The agent may be systemic after administration or may be localized by the use of an implant that acts to retain the active dose at the site of implantation.

In pharmaceutical dosage forms, the compounds may be administered in the form of their pharmaceutically acceptable salts, or they may also be used alone or in appropriate association, as well as in combination with other pharmaceutically active compounds. The following methods and excipients are merely exemplary and are in no way limiting.

For oral preparations, the compounds can be used alone or in combination with appropriate additives to make tablets, powders, granules or capsules, for example, with conventional additives, such as lactose, mannitol, corn starch or potato starch; with binders, such as crystalline cellulose, cellulose derivatives, acacia, corn starch or gelatins; with disintegrators, such as corn starch, potato starch or sodium carboxymethylcellulose; with lubricants, such as talc or magnesium stearate; and if desired, with diluents, buffering agents, moistening agents, preservatives and flavoring agents.

The compounds can be formulated into preparations for injections by dissolving, suspending or emulsifying them in an aqueous or nonaqueous solvent, such as vegetable or other similar oils, synthetic aliphatic acid glycerides, esters of higher aliphatic acids or propylene glycol; and if desired, with conventional additives such as solubilizers, isotonic agents, suspending agents, emulsifying agents, stabilizers and preservatives.

The compounds can be utilized in aerosol formulation to be administered via inhalation. The compounds of the present invention can be formulated into pressurized acceptable propellants such as dichlorodifluoromethane, propane, nitrogen and the like.

Furthermore, the compounds can be made into suppositories by mixing with a variety of bases such as emulsifying bases or water-soluble bases. The compounds of the present invention can be administered rectally via a suppository. The suppository can include vehicles such as cocoa butter, carbowaxes and polyethylene glycols, which melt at body temperature, yet are solidified at room temperature.

Unit dosage forms for oral or rectal administration such as syrups, elixirs, and suspensions may be provided wherein each dosage unit, for example, teaspoonful, tablespoonful, tablet or suppository, contains a predetermined amount of the composition containing one or more compounds of the present invention. Similarly, unit dosage forms for injection or intravenous
5 administration may comprise the compound of the present invention in a composition as a solution in sterile water, normal saline or another pharmaceutically acceptable carrier.

Implants for sustained release formulations are well-known in the art. Implants are formulated as microspheres, slabs, *etc.* with biodegradable or non-biodegradable polymers. For example, polymers of lactic acid and/or glycolic acid form an erodible polymer that is well-tolerated
10 by the host. The implant is placed in proximity to the site of disease, so that the local concentration of active agent is increased relative to the rest of the body.

The term "unit dosage form," as used herein, refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of compounds of the present invention calculated in an amount sufficient to produce the desired effect
15 in association with a pharmaceutically acceptable diluent, carrier or vehicle. The specifications for the novel unit dosage forms of the present invention depend on the particular compound employed and the effect to be achieved, and the pharmacodynamics associated with each compound in the host.

The pharmaceutically acceptable excipients, such as vehicles, adjuvants, carriers or diluents, are readily available to the public. Moreover, pharmaceutically acceptable auxiliary substances, such
20 as pH adjusting and buffering agents, tonicity adjusting agents, stabilizers, wetting agents and the like, are readily available to the public.

Typical dosages for systemic administration range from 0.1 μ g to 100 milligrams per kg weight of subject per administration. A typical dosage may be one tablet taken from two to six times daily, or one time-release capsule or tablet taken once a day and containing a proportionally higher
25 content of active ingredient. The time-release effect may be obtained by capsule materials that dissolve at different pH values, by capsules that release slowly by osmotic pressure, or by any other known means of controlled release.

Those of skill will readily appreciate that dose levels can vary as a function of the specific compound, the severity of the symptoms and the susceptibility of the subject to side effects. Some of
30 the specific compounds are more potent than others. Preferred dosages for a given compound are readily determinable by those of skill in the art by a variety of means. A preferred means is to measure the physiological potency of a given compound.

The use of liposomes as a delivery vehicle is one method of interest. The liposomes fuse with the cells of the target site and deliver the contents of the lumen intracellularly. The liposomes are
35 maintained in contact with the cells for sufficient time for fusion, using various means to maintain contact, such as isolation, binding agents, and the like. In one aspect of the invention, liposomes are designed to be aerosolized for pulmonary administration. Liposomes may be prepared with purified proteins or peptides that mediate fusion of membranes, such as Sendai virus or influenza virus, *etc.* The lipids may be any useful combination of known liposome forming lipids, including cationic lipids,

such as phosphatidylcholine. The remaining lipid will normally be neutral lipids, such as cholesterol, phosphatidyl serine, phosphatidyl glycerol, and the like.

MODULATION OF ENZYME ACTIVITY

5 Agents that block activity of cancer associated kinase corresponding to SEQ ID NOS:1, 3, 5, 7, 9, 11 or 13 provide a point of intervention in an important signaling pathway. Numerous agents are useful in reducing this activity, including agents that directly modulate expression as described above, e.g. expression vectors, antisense specific for the targeted kinase; and agents that act on the protein, e.g. specific antibodies and analogs thereof, small organic molecules that block catalytic
10 activity, etc.

The genes, gene fragments, or the encoded protein or protein fragments are useful in therapy to treat disorders associated with defects in sequence or expression. From a therapeutic point of view, inhibiting activity has a therapeutic effect on a number of proliferative disorders, including inflammation, restenosis, and cancer. Inhibition is achieved in a number of ways.
15 Antisense sequences may be administered to inhibit expression. Pseudo-substrate inhibitors, for example, a peptide that mimics a substrate for the kinase may be used to inhibit activity. Other inhibitors are identified by screening for biological activity in a functional assay, e.g. *in vitro* or *in vivo* kinase activity.

Expression vectors may be used to introduce the target gene into a cell. Such vectors
20 generally have convenient restriction sites located near the promoter sequence to provide for the insertion of nucleic acid sequences. Transcription cassettes may be prepared comprising a transcription initiation region, the target gene or fragment thereof, and a transcriptional termination region. The transcription cassettes may be introduced into a variety of vectors, e.g. plasmid; retrovirus, e.g. lentivirus; adenovirus; and the like, where the vectors are able to transiently or stably
25 be maintained in the cells, usually for a period of at least about one day, more usually for a period of at least about several days to several weeks.

The gene or protein may be introduced into tissues or host cells by any number of routes, including viral infection, microinjection, or fusion of vesicles. Jet injection may also be used for intramuscular administration, as described by Furth *et al.* (1992) Anal Biochem 205:365-368. The
30 DNA may be coated onto gold microparticles, and delivered intradermally by a particle bombardment device, or "gene gun" as described in the literature (see, for example, Tang *et al.* (1992) Nature 356:152-154), where gold micro projectiles are coated with the protein or DNA, then bombarded into skin cells.

Antisense molecules can be used to down-regulate expression in cells. The antisense
35 reagent may be antisense oligonucleotides (ODN), particularly synthetic ODN having chemical modifications from native nucleic acids, or nucleic acid constructs that express such antisense molecules as RNA. The antisense sequence is complementary to the mRNA of the targeted gene, and inhibits expression of the targeted gene products. Antisense molecules inhibit gene expression through various mechanisms, e.g. by reducing the amount of mRNA available for translation, through

activation of RNase H, or steric hindrance. One or a combination of antisense molecules may be administered, where a combination may comprise multiple different sequences.

Antisense molecules may be produced by expression of all or a part of the target gene sequence in an appropriate vector, where the transcriptional initiation is oriented such that an antisense strand is produced as an RNA molecule. Alternatively, the antisense molecule is a synthetic oligonucleotide. Antisense oligonucleotides will generally be at least about 7, usually at least about 12, more usually at least about 20 nucleotides in length, and not more than about 500, usually not more than about 50, more usually not more than about 35 nucleotides in length, where the length is governed by efficiency of inhibition, specificity, including absence of cross-reactivity, and the like. It has been found that short oligonucleotides, of from 7 to 8 bases in length, can be strong and selective inhibitors of gene expression (see Wagner *et al.* (1996) Nature Biotechnology 14:840-844).

A specific region or regions of the endogenous sense strand mRNA sequence is chosen to be complemented by the antisense sequence. Selection of a specific sequence for the oligonucleotide may use an empirical method, where several candidate sequences are assayed for inhibition of expression of the target gene *in vitro* or in an animal model. A combination of sequences may also be used, where several regions of the mRNA sequence are selected for antisense complementation.

Antisense oligonucleotides may be chemically synthesized by methods known in the art (see Wagner *et al.* (1993) *supra.* and Milligan *et al.*, *supra.*) Preferred oligonucleotides are chemically modified from the native phosphodiester structure, in order to increase their intracellular stability and binding affinity. A number of such modifications have been described in the literature, which alter the chemistry of the backbone, sugars or heterocyclic bases.

Among useful changes in the backbone chemistry are phosphorothioates; phosphorodithioates, where both of the non-bridging oxygens are substituted with sulfur; phosphoroamidites; alkyl phosphotriesters and boranophosphates. Achiral phosphate derivatives include 3'-O'-5'-S-phosphorothioate, 3'-S-5'-O-phosphorothioate, 3'-CH₂-5'-O-phosphonate and 3'-NH-5'-O-phosphoroamidate. Peptide nucleic acids replace the entire ribose phosphodiester backbone with a peptide linkage. Sugar modifications are also used to enhance stability and affinity. The alpha.-anomer of deoxyribose may be used, where the base is inverted with respect to the natural .beta.-anomer. The 2'-OH of the ribose sugar may be altered to form 2'-O-methyl or 2'-O-allyl sugars, which provides resistance to degradation without comprising affinity. Modification of the heterocyclic bases must maintain proper base pairing. Some useful substitutions include deoxyuridine for deoxythymidine; 5-methyl-2'-deoxycytidine and 5-bromo-2'-deoxycytidine for deoxycytidine. 5-propynyl-2'-deoxyuridine and 5-propynyl-2'-deoxycytidine have been shown to increase affinity and biological activity when substituted for deoxythymidine and deoxycytidine, respectively.

EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

The present invention has been described in terms of particular embodiments found or proposed by the present inventor to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of the present disclosure, numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. For example, due to codon redundancy, changes can be made in the underlying DNA sequence without affecting the protein sequence. Moreover, due to biological functional equivalency considerations, changes can be made in protein structure without affecting the biological action in kind or amount. All such modifications are intended to be included within the scope of the appended claims.

Example 1MAP3K11

The Genbank database was searched for ESTs showing similarity to known kinase domain-related proteins using the "basic local alignment search tool" program, TBLASTN, with default settings. Human ESTs identified as having similarity to these known kinase domain (defined as $p < 0.0001$) were used in a BLASTN and BLASTX screen of the GenBank non-redundant (NR) database.

ESTs that had top human hits with >95% identity over 100 amino acids were discarded. This was based upon the inventors' experience that these sequences were usually identical to the starting probe sequences, with the differences due to sequence error. The remaining BLASTN and BLASTX outputs for each EST were examined manually, i.e., ESTs were removed from the analysis if the inventors determined that the variation from the known kinase domain -related probe sequence was a result of poor database sequence. Poor database sequence was usually identified as a number of 'N' nucleotides in the database sequence for a BLASTN search and as a base deletion or insertion in the database sequence, resulting in a peptide frameshift, for a BLASTX output. ESTs for which the highest scoring match was to non-kinase domain-related sequences were also discarded at this stage.

Using widely known algorithms, e.g. "Smith/Waterman", "Fasta", "FastP", "Needleman/Wunsch", "Blast", "PSIBlast," homology of the subject nucleic acid to other known nucleic acids was determined. A "Local FastP Search" algorithm was performed in order to

determine the homology of the subject nucleic acid invention to known sequences. Then, a ktup value, typically ranging from 1 to 3 and a segment length value, typically ranging from 20 to 200, were selected as parameters. Next, an array of position for the probe sequence was constructed in which the cells of the array contain a list of positions of that substring of length ktup. For each
5 subsequence in the position array, the target sequence was matched and augmented the score array cell corresponding to the diagonal defined by the target position and the probe subsequence position. A list was then generated and sorted by score and report. The criterion for perfect matches and for mismatches was based on the statistics properties of that algorithm and that database, typically the values were: 98% or more match over 200 nucleotides would constitute a match; and any mismatch
10 in 20 nucleotides would constitute a mismatch.

Analysis of the BLASTN and BLASTX outputs identified a EST sequence from IMAGE clone AI803752 that had potential for being associated with a sequence encoding a kinase domain-related protein, e.g., the sequence had homology, but not identity, to known kinase domain-related proteins.

After identification of MAP3K11 ESTs were discovered, the clones were added to Kinetek's
15 clone bank for analysis of gene expression in tumor samples. Gene expression work involved construction of unigene clusters, which are represented by entries in the "pks" database. A list of accession numbers for members of the clusters were assigned. Subtraction of the clusters already present in the clone bank from the clusters recently added left a list of clusters that had not been previously represented in Kinetek's clone bank. For each of the clusters, a random selection of an
20 EST IMAGE accession numbers were chosen to keep the clusters. For each of the clusters which did not have an EST IMAGE clone, generation of a report so that clone ordering or construction could be implemented was performed on a case by case basis. A list of accession numbers which were not in clusters was constructed and a report was generated.

The AI803752 IMAGE clone was sequenced using standard ABI dye-primer and
25 dye-terminator chemistry on a 377 automatic DNA sequencer. Sequencing revealed that the sequence corresponds to SEQ ID NO:1.

Rapid Amplification of cDNA Ends (RACE).

The gene specific oligodeoxynucleotide primers SEQ ID NO:15 and 16 were designed and
30 then used to construct full length MAP3K11 cDNA by 5 prime RACE (rapid amplification of cDNA ends; Frohman *et al.* (1988), Proc. Natl. Acad. Sci. USA 85:8898-9002).

A nested primer strategy was used on human brain cDNA provided with a Marathon-Ready™ RACE kit (Clontech, Palo Alto, CA). Following this, thermal cycling on a PE DNA Thermal Cycler 480 was done. When cycling was completed, the PCR product was analyzed, along
35 with appropriate DNA size markers, on a 1.0% agarose/EtBr gel.

The product so obtained comprised a MAP3K11 polynucleotide having the sequence of SEQ ID NO:1.

Expression Analysis of MAP3K11

The expression of MAP3K11 was determined by dot blot analysis, and the protein was found to be upregulated in several tumor samples.

Dot blot preparation. Total RNA was purified from clinical cancer and control samples taken from the same patient. Samples were used from both liver and colon cancer samples. Using reverse transcriptase, cDNAs were synthesized from these RNAs. Radiolabeled cDNA was synthesized using Strip-EZ™ kit (Ambion, Austin, TX) according to the manufacturer's instructions. These labeled, amplified cDNAs were then used as a probe, to hybridize to human protein kinase arrays comprising human MAP3K11. The amount of radiolabeled probe hybridized to each arrayed EST clone was detected using phosphorimaging.

The expression of MAP3K11 was substantially upregulated in the tumor tissues that were tested. The data is shown in Table 1, expressed as the fold increase over the control non-tumor sample.

Table 1

Target	liver 1	liver 2	liver 3	colon 1	colon 4	colon 5	colon 7	colon 8	colon 9	colon 10
MAP3K11	4.1	1.3	2.3	2.1	1.1	1.9	3.4	1.3	0.9	1.75
beta-actin	2.05	1.07	1.57	0.42	1.28	2.19	1.20	4.60	0.60	0.49
GAPDH	1.30	0.33	1.25	0.76						
K413 (ribosomal protein)					1.72	2.36	2.10	1.00	1.00	1.68

The data displayed in Table 2 provides a brief summary of the pathology report of the patient samples.

Table 2

Patient	Age	Gender	Precursor Adenoma	Site of Involvement	Differentiation	Vascular Invasion	Lymphatic Involvement	Metastasis
Liver 1	49	Female	N/a	Liver	Moderately Differentiated	No	Yes	No
Liver 2	53	Male	N/a	Liver	Moderately Differentiated	Yes	No	No
Liver 3	75	Female	Adenoma	Right Colon	Moderately differentiated	No	No	No
Colon 1	55	Female	No	Rectum	Moderately Differentiated	N/A	Yes	No
Colon 4	91	Female	Adenoma	Cecum	Moderately Differentiated	No	Yes	No
Colon 5	79	Male	No	Ileum and Colon				
Colon 7					Moderately Differentiated	No	No	No
Colon 8	61	Male	Yes		Moderately Differentiated	No	Yes	Yes, Liver
Colon 9	60	Male	No	Recto-Sigmoid	Moderately Differentiated	Yes	No	Yes, Liver
Colon	60	Male	No	Sigmoid	Moderately	Yes	Yes	No

10				Colon	Differentiated			
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Example 2

CaMK-X1

The Genbank database was searched for ESTs showing similarity to known kinase domain-related proteins using the "basic local alignment search tool" program, TBLASTN, with default settings. Human ESTs identified as having similarity to these known kinase domain (defined as $p < 0.0001$) were used in a BLASTN and BLASTX screen of the GenBank non-redundant (NR) database, searched against the sequence of the catalytic domain of CaMK-I (Genbank hs2721161). Sequence screening was performed as described in Example 1.

Analysis of the BLASTN and BLASTX outputs identified an EST sequence from IMAGE clone AA838372 that had potential for being associated with a sequence encoding a kinase domain-related protein, e.g., the sequence had homology, but not identity, to known kinase domain-related proteins. Further, CaMK-X1 was found to have sequence similarity to members of the calmodulin dependent protein kinase family. The reported nucleotide sequence of the 5' EST of the AA838372 IMAGE clone corresponds approximately to 400 nucleotides of SEQ ID NO:1. A search of the UniGene database revealed that the 5' EST of the AA838372 IMAGE clone represented a novel human gene.

The AA838372 IMAGE clone was sequenced using standard ABI dye-primer and dye-terminator chemistry on a 377 automatic DNA sequencer. Sequencing revealed that the sequence corresponds to nucleotides 1 to 2447 of SEQ ID NO:3. Analysis of this gene fragment revealed that the gene product is a novel kinase domain-related protein, thereafter termed CaMK-X1.

Rapid Amplification of cDNA Ends (RACE).

The gene specific oligodeoxynucleotide primer 5'-GGAGGGCG AGGAAACTGGGGAAG -3' (SEQ ID NO:17) was designed and then used to construct full length CaMK-X1 cDNA by 5 prime RACE (rapid amplification of cDNA ends; Frohman *et al.* 1988, Proc. Natl. Acad. Sci. USA 85:8898-9002). Adaptor primer (AP1) was used as sense primer, and SEQ ID NO:3 was used as antisense primer. A nested primer strategy was used on fetal brain cDNA provided with a Marathon-Ready™ RACE kit (Clontech, Palo Alto, CA). Following this, thermal cycling on a PE DNA Thermal Cycler 480 was done. When cycling was completed, the PCR product was analyzed, along with appropriate DNA size markers, on a 1.0% agarose/EtBr gel.

The product so obtained comprised a CaMK-X1 polynucleotide having the sequence of SEQ ID NO:3. BLASTX analysis indicated that the starting methionine residue was present at nucleotide 10, and that an upstream in-frame stop codon was present at nucleotide 1498, and the longest ORF (SEQ ID NO:3) predicted a protein of 476 amino acids (SEQ ID NO:4).

Homology analysis of the deduced amino acid sequence of CaMK-X1 revealed strong sequence identity with CaMK I from amino acid residues 11 to 333. The corresponding region of CaMK I contains the threonine residue required for activation and the regulatory domain that folds over the active site unless bound by CaM (Matsuchita *et al.* (1998) *Journal of Biological Chemistry*

273, 21473-21481). CaMK-X1 also has a region between residues 23 and 277 that is highly homologous (46% identity) to the highly conserved serine/threonine kinase active site.

Expression Analysis

5 The expression of CaMK-X1 was determined by Northern Blot, and dot blot analysis, and the protein was found to be upregulated in several tumor samples. In normal tissue, CaMK-X1 is highly expressed in brain, and at lower levels in kidney and spleen.

10 *Dot blot preparation.* Total RNA was purified from clinical cancer and control samples taken from the same patient. Samples were used from both liver and colon cancer samples. Using reverse transcriptase, cDNAs were synthesized from these RNAs. Radiolabeled cDNA was synthesized using Strip-EZ™ kit (Ambion, Austin, TX) according to the manufacturer's instructions. These labeled, amplified cDNAs were then used as a probe, to hybridize to human protein kinase arrays comprising human CaMK-X1. The amount of radiolabeled probe hybridized to each arrayed EST clone was detected using phosphorimaging.

15 The expression of CaMK-X1 was substantially upregulated in the tumor tissues that were tested. The data is shown in Table 3, expressed at the fold increase over the control non-tumor sample.

Table 3

	liver 1	liver 2	liver 3	colon 1	colon 2	colon 3	colon 4	colon 5	colon 6	colon 7
CaMK-X1	5.0	4.9	5.1	2.3	2.6	1.5	3.3	1.2	1.3	4.05

Functional Assays

A deletion mutant clone was created to aid in the characterization of this kinase *in vivo*. In addition, it is shown that CaMK-X1 phosphorylates CREB at Ser 133 in Jurkat cells, and this phosphorylation is controlled by a Calmodulin binding site.

25 CaMK-X1 kinase activity was shown *in vitro* using three different approaches. CaMK-X1 was purified from Hi5 insect cells and HEK293 cells overexpressing CaMK-X1 using GST and Ni²⁺ affinity chromatography. Furthermore, CaMK-X1 was purified via immunoprecipitation using a monoclonal antibody directed against the X-press fusion protein. CaMK-X1 displays no activity toward exogenous substrates in the absence of Ca²⁺ and calmodulin. In the presence of Ca²⁺ and calmodulin, CaMK-X1 phosphorylated Syntide and CREBtide peptides. This is the first experimental demonstration that CaMK-X1 behaves as a calcium/calmodulin-dependent protein kinase.

35 *Cloning and sub-cloning.* Cloning of CaMK-X1 and construction of cDNA expression vectors and the CaMK-X1 deletion mutant: A human brain cDNA library was used with a 5' RACE system. To generate the full-length cDNA of CaMK-X1, a pair of primers were designed and used in the PCR reaction. (SEQ ID NO:24) 5'-GTGGAGGGC GAGGAACTGGGGAAG-3 and (SEQ ID NO:25) 5'-CTCGAGTCACA TAATGAGACAGACTCCAGTC. The coding area of CaMK-X1 was amplified using

the above pair of primers. The amplification product was then cloned into a Promega T/A vector and subsequently cloned into other vectors as necessary. The EcoRI and XhoI fragment of CaMK-X1 was cloned into bacterial expression vector pGEX-4T-3 and mammalian expression vector pcDNA3.1/His B. All constructs were verified by restriction enzyme digestion and DNA sequencing.

5

Tissue distribution of CaMK-X1. CaMK-X1 was used to probe and blot mRNA, using a commercially available poly-A⁺ selected blot (Clontech, Palo Alto, CA), and hybridized according to the manufacturer's instructions. The CaMK-X1 clone (corresponding to SEQ ID NO:3) was radiolabeled using Strip-EZ PCR kit (Ambion, Austin, TX) according to the manufacturer's instructions.

10

It was found that in normal tissues, CaMK-X1 is expressed at high levels only in the brain, hybridizing to an mRNA of approximately 2.8 Kb in length. The mRNA was expressed at low levels in the kidney and spleen. The mRNA in the Northern blot ran at a position consistent with a molecular weight between 2.5-2.7 kb.

15

CaMK-X1 increases proliferation of Cos7 cells. The proliferation rate of Cos7 cells when transfected with CaMK-X1 was examined. To determine whether increased levels of CaMK-X1 had any effect on cell proliferation, Cos7 cells were transfected with increasing concentrations of CaMK-X1 or vector plasmids in the presence of KCl. Cell proliferation was measured by standard protocols. As shown in Fig. 1, transfection of CaMK-X1 increased the rate of proliferation, whereas the same concentration of vector alone decreased the rate of proliferation. The proliferation rate of Cos7 cells transiently transfected with CaMK-X1 is higher in 5% serum than at the 2.5% or 0.5%, suggesting that CaMK-X1 induced proliferation is modulated by serum. This data demonstrates that CaMK-X1 can promote cell proliferation.

20

CaMK-X1 phosphorylates CREB in vivo. cAMP response element-binding protein (CREB) is a DNA binding transcription factor. A number of growth factors and hormones have been shown to stimulate the expression of cellular genes by inducing the phosphorylation of the nuclear factor CREB at Ser 133 (Montminy (1997) Annu.Rev. Biochem. 66:807-822). Originally characterized as a target for PKA-mediated phosphorylation, CREB is also recognized by other kinases including Protein kinase C, calmodulin kinase, microtubule-activated protein kinase activated protein, and protein kinase B/AKT.

25

It was investigated whether CaMK-X1 could regulate CREB-Ser 133 phosphorylation *in vivo*. To analyze CaMK-X1 *in vivo*, Jurkat cells were utilized. Jurkat cells transfected with various concentrations of plasmids carrying CaMK-X1 or vector were stimulated with KCl. Whole cell protein was prepared from these transfected cells and the phosphorylation status of CREB at Ser 133 was determined. Detection of CREB phosphorylation was carried out using anti-phospho-CREB antibody. Phosphorylation of CREB increased with increasing amounts of the CaMK-X1 gene transfection, but only in the presence of Ca²⁺.

30

To assess the effects of intracellular Ca^{2+} on CaMK-X1, transfected Jurkat cells were treated with 30 mM KCl. KCl depolarizes cell membranes thereby creating an increase in intracellular Ca^{2+} . Addition of KCl resulted in significant phosphorylation of CREB only in cells transfected with CaMK-X1. These results show that CaMK-X1 is activated by Ca^{2+} and subsequently phosphorylates CREB at Ser 133 in Jurkat cells.

Calmodulin binding site deletion mutant of CaMK-X1 constitutively phosphorylates CREB in vivo. It has been shown previously that CaM kinases can be made Ca^{2+} independent by truncation of the calmodulin binding site. Similarly, a constitutively active form of CaMK-X1 was created by removing the putative CaM-binding domain via truncation at amino acid Gln 301. This deletion site eliminates the two predicted Ca^{2+} /Calmodulin-binding sites in the autoinhibitory domain. The truncated gene was placed in a pcDNA mammalian expression vector for transfection experiments.

To analyze the function of the mutant CaMK-X1 *in vivo*, Jurkat cells were used. Jurkat cells transfected with various concentrations of plasmids carrying CaMK-X1 or vector were stimulated with KCl. Whole cell protein was prepared from these transfected cells and the phosphorylation status of CREB at Ser 133 was determined. Detection of CREB phosphorylation was carried out using anti-phospho-CREB antibody. Mock treatment by the vectors did not have any effect on CREB phosphorylation. The transfection of wild type CaMK-X1 had no effect on CREB phosphorylation; however, addition of KCl to wild type transfected Jurkat cells resulted in significant CREB phosphorylation. Transfection of the deletion mutant had a significant effect on CREB phosphorylation without the addition of KCl. These results demonstrate that truncation of wild type CaMK-X1 at Gln 301 converted the enzyme to a Ca^{2+} /CaM-independent state.

Expression of CaMK-X1 kinase in HEK293 cells. The availability of the CaMK-X1 clone allows us to reconstruct the signaling pathway. This allows us to identify downstream components such as transcription factors or modification of protein components such as phosphorylation, proteolytic processing, methylation, and the like, which finds use in drug screening.

To characterize CaMK-X1 at the protein level, HEK293 cells were transfected with pcDNA3-Xpress (Invitrogen) containing the CaMK-X1 coding sequence fused to the Xpress epitope; and stable cell lines were created using standard techniques. Five stable cell lines containing the pcDNA-CaMK-X1 plasmid and five containing the vector only control were selected and CaMK-X1 expression levels were determined. Whole cell extracts were prepared from each cell line. The cell lysates were analysed by Western blotting with an anti Xpress monoclonal antibody. These experiments revealed a 53 kDa fusion protein present in the CaMK-X1 transfected cells that was absent in the control cells.

The transfected HEK293 cells stably expressed CaMK-X1 as an Xpress fusion protein. Similarly, we have detected a GST-CaMK-X1 fusion protein expressed in Hi5 cells. Glutathione-sepharose affinity chromatography was used to purify the GST-CaMK-X1 fusion protein. Glutathione-sepharose purified CaMK-X1 and anti-Xpress antibody immunoprecipitated CaMK-X1 were subjected to Western blot analysis. This Western blot indicates that CaMK-X1 can be purified

from both transfected HEK293 cell lysate and Hi5 cell lysate. These methodologies were used to purify CaMK-X1 for further characterization.

5 A protein with a molecular mass of 53kDa was identified when lysates of HEK293 cells transfected with the Xpress-CaMK-X1 clone were subjected to immunoprecipitation with anti-Xpress antibody followed by anti-Xpress Western blotting, which band was absent with vector alone transfected cells. This data confirms that the anti-Xpress antibody selectively immunoprecipitated the fusion protein (Xpress-CaMK-X1).

10 These immunoprecipitated materials were assayed for kinase activity, using the peptides (SEQ ID NO:26) CREBtide: Lys Arg Arg Glu Ile Leu Ser Arg Arg Pro Ser Tyr Arg; (SEQ ID NO:27) Syntide 2: Pro Leu Ala Arg Thr Leu Ser Val Ala Gly Leu Pro Gly Lys Lys; and (SEQ ID NO:28) Calmodulin Dependent Protein Kinase Substrate: Pro Leu Ser Arg Thr Leu Ser Val Ser Ser. The immunoprecipitated materials were subjected to an *in vitro* kinase assay as described above. Since it was shown that CaMK-X1 phosphorylates CREB *in vivo*, it was reasoned that CaMK-X1 would phosphorylate CREBtide and Syntide 2 (Colbran *et al.* (1989) J Biol Chem 264(9):4800-4804). As
15 predicted, CaMK-X1 phosphorylated CREBtide and Syntide 2 *in vitro*. In contrast, CaMK-X1 could not phosphorylate control peptide. The degree of phosphorylation is augmented in the presence of calmodulin, as shown in Figure 2. In the absence of a substrate, there is no significant incorporation of radioactive material (^{32}P) indicating that CaMK-X1 does not autophosphorylate under these assay conditions. This demonstrates that immunoprecipitated CaMK-X1 possesses a kinase activity and
20 that this kinase activity is capable of phosphorylating peptides *in vitro*. These studies also revealed that CaMK-X1 requires calmodulin for efficient activity.

Catalytic activity and comparison of substrate specificities of CaMK-X1. In order to determine if CaMK-X1 is an active kinase *in vitro*, the clone was Histidine tagged, expressed in Sf9
25 cells and purified with a Ni^{2+} affinity column. For analysis of substrate specificity, we tested the following three peptides; CREBtide, Syntide 2 and CDPK-peptide (control peptide). *In vitro* kinase assays were then performed. As described above, CREBtide and Syntide 2 are phosphorylated by the purified CaMK-X1. The rate of phosphorylation is increased in the presence of Ca^{2+} and calmodulin. Compared to a no substrate control, addition of the peptides resulted in significant ^{32}P
30 incorporation. These results indicate that CaMK-X1 phosphorylates these peptides *in vitro*. Our studies also revealed Syntide 2 and CREBtide had higher incorporation of ^{32}P than the control peptide. These observations further confirm the *in vivo* data.

Summary. We have demonstrated that CaMK-X1 phosphorylates CREB in cells and *in vitro*
35 at Ser 133. We have also demonstrated CaMK-X1 kinase activity *in vitro*. We were able to purify CaMK-X1 from transfected Hi5 insect cells and from a HEK293 cell line overexpressing CaMK-X1 using glutathione-sepharose and Ni^{2+} affinity chromatography. Furthermore, CaMK-X1 was purified by immunoprecipitation using a monoclonal antibody directed against the Xpress fusion protein. CaMK-X1 displays no activity toward exogenous substrates in the absence of Ca^{2+} and calmodulin.

In the presence of Ca^{2+} and calmodulin, CaMK-X1 phosphorylated Syntide 2 and CREBtide. These results indicate that Camk X-1 are involved in human pathology.

Materials.

5 Dulbecco's Modified Eagle Medium (DMEM), RPMI Medium 1640, L-glutamine, phosphate buffered solution (PBS), fetal bovine serum (FBS), and restriction enzymes were from GibcoBRL. TOPO cloning kit (including PCR materials and pCR 2.1-Topo vector) were from Invitrogen. Phospho-CREB (Ser133) polyclonal rabbit antibody was from Cell Signaling Technology. 96- and 6-
10 well delta surface plates were from NUNCCLON. QIAprep Spin Miniprep Kit was from Qiagen. Wizard Plus Minipreps DNA Purification System (for gel extractions) (Promega). FuGENE 6 Transfection Reagent was from Boehringer Mannheim. pcDNA3.1 mammalian expression vector (Invitrogen). Western Blotting Luminol Reagent was from Santa Cruz Biotechnology. 2° goat-anti-rabbit IgG (H+L) HRP conjugated antibody was from Bio-Rad Laboratories.

Cloning of full length CaMK-X1. To generate the full-length cDNA of CaMK-X1, a pair of
15 primers were designed and used in the PCR reaction. (SEQ ID NO:29) 5'-GAATTCAATGGGTCGAAAGGAAGAAGATGA and (SEQ ID NO:25) 5'-CTCGAGTCACATAATGAGACAGACTCCAGTC. The amplification product was cloned into cloning vectors through restriction sites EcoRI and XhoI. The EcoRI and XhoI fragment was cloned into bacteria expression vector pGEX-4T-3 and mammalian expression vector pcDNA3.1/HisB. All
20 constructs were verified by restriction enzyme digestion and DNA sequencing.

Construction of deletion mutant CaMK-X1CA. A deletion mutant was created using these oligonucleotides EcoR1 (SEQ ID NO:30) 5'-GAATTCAATGGGTCGAAAGGAAGAAGATGA-3' forward, and Xho1 (SEQ ID NO:31) 5'-CTCGAGCTGGATCTGGAGGCTGACTGATGG-3' reverse. The resulting PCR fragment was cloned into mammalian expression vector pcDNA 3.1.

25 *Cell Culture.* Cells were incubated at 37°C in 5% CO₂ (standard conditions). All cells, unless mentioned below, were cultured in DMEM with FBS; the specific amount of FBS varies and is stated in the report for each result. Jurkat cells were cultured in RPMI Medium 1640 with added glucose, L-glutamine, and 10% FBS.

Cell Transfection. Cells were seeded to a density of 2×10^5 in 6 well plates (in appropriate
30 media for the particular cell line) and incubated for 24 hours under standard conditions. 3 ml of FuGENE 6 transfection reagent was diluted in 97 ml of serum-free media (appropriate for the cell line being transfected) and left for 5 minutes at room temperature; that was then added drop-wise to the desired amount of plasmid DNA (in pcDNA3.1) and left for 10 minutes at room temperature. The finished transfection solution was then added drop-wise to the cells, which were then incubated for
35 24 hours under standard conditions.

Proliferation Assay. The media from 6 well plates was removed and trypsin was added to digest the extracellular matrix holding the cells to the plate; media (appropriate to the cell type) was then added to deactivate the trypsin. The cells and media were transferred into Falcon tubes, centrifuged, and the supernatant was discarded. The cells were resuspended in appropriate media.
40 3000 cells were seeded in each well of a 96 well plate and appropriate media was added up to 90 ml.

Ten μ l of 0.1 Ci/L 3 H-thymidine was added to each well. The plates were then incubated for 24 hours under standard conditions. Twenty-five μ l of cold trichloroacetic acid was added to each well and the plates were kept at 4°C for 2 hours. The plates were then washed in cold running water and allowed to dry. Proliferation was determined by incorporation of thymidine as measured via scintillation counting.

Cell lysis. Lysis buffer was 50 mM Hepes (pH 7.5), 150 mM NaCl, 1% NP-40, 2 mM NaF, 1mM Na_3VO_4 , 1mM PMSF, 1 mg/ml pepstatin, 1 mg/ml leupeptin, 1 mg/ml aprotinin, and 20 mM β -glycerophosphate. For adherent cells, the media was removed from the 6 well plate, the wells were washed with PBS which was then removed, the plates were put on ice and 40 ml of lysis buffer was then added to each well. Crude lysates were collected with a cell scraper and placed in an Eppendorf tube. For non-adherent cells, the media and cells were transferred from a 6-well plate to tubes, centrifuged and the supernatant removed; 40 ml of lysis buffer was then added. All crude lysates were then vortexed and left on ice for 10 minutes. The crude lysates were centrifuged at 14,000 RPM for 10 minutes at 4°C and the supernatant, the final lysate, was transferred to new tubes.

Western Blotting. Equal weights of cell lysate proteins were mixed with 4X loading buffer, boiled for five minutes and were then briefly centrifuged. The samples were run on a 10% SDS-PAGE and were then transferred to PVDF membranes which were washed with TTBS and blocked with 2% BSA. They were blotted with primary antibody for 16 hours at 4°C. The membranes were washed with TTBS, blotted with secondary antibody for 1 hour and washed with TTBS. The luminol reagent was added, the blot was placed on film and the autoradiograph developed.

Expression and purification of CaMK-X1 protein. The human CaMK-XI gene (K283) was sub-cloned into baculovirus transfer vector pAcG4T3 derived from pAcG2T (BD Biosciences) under the control of the strong AcNPV (*Autographa californica* Nuclear Polyhedrosis Virus) polyhedrin promoter. This was co-transfected with linear BaculoGold DNA in *Spodoptera frugiperda* Sf9 cells following standard procedure (BD Biosciences). The GST-CaMK-X1 recombinant baculovirus was amplified in Sf9 cells in TNM-FH medium (JHR Biosciences) with 10% fetal bovine serum. The GST-CaMK-X1 protein was expressed in approximately 5×10^8 Hi-5 cells (Invitrogen) in 500 ml of Excell-400 medium (JHR Biosciences) at a multiplicity of infection (MOI) of five for a period of 72 h in a spinner flask. The cells were harvested at 800Xg for 5 min at 4°C. The pellet was lysed in 40 ml of Lysis Buffer (50 mM Tris-HCl, PH7.5, 2.5 mM EDTA, 150 mM NaCl, 1% NP-40, 0.1% β -mercaptoethanol, 10 μ g/ml DNase I, 0.5 mM sodium orthovanadate, 50 mM β -glycerophosphate, 0.1 mM PMSF, 1 mM benzamidine, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 1 μ g/ml pepstatin) by sonication and centrifuged at 10,000Xg at 4°C for 15 min. The supernatant was loaded on a column containing 2.5 ml of glutathione-sepharose (Sigma). The column was washed with Wash Buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 500 mM NaCl, 0.1% β -mercaptoethanol, 0.1% NP-40, 0.1 mM sodium orthovanadate, 50 mM β -glycerophosphate, 0.1 mM PMSF, 1 mM benzamidine) until OD280 returned to baseline, then Wash Buffer B (50 mM Tris-HCl, PH7.5, 1 mM EDTA, 50 mM NaCl, 0.1% β -mercaptoethanol, 0.1 mM PMSF). The GST-CaMK-X1 protein was eluted in Elution Buffer (50 mM

Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl, 0.1% β -mercaptoethanol, 10 mM glutathione, 10% glycerol). The fraction was aliquoted and stored at -70°C .

5 *CaMK-X1 in vitro assay.* CaMK-X1 was assayed at room temperature for 15 min in 50 mM HEPES, pH 8.0, 10 mM MgCl_2 , 1 mM dithiothreitol, 0.005% Tween 20, 1 mM CaCl_2 , 1.5 mM calmodulin (CalBiochem), 50 μM $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ and 0.2 $\mu\text{g}/\mu\text{l}$ Syntide 2 (American Peptide Company) or CREBtide (CalBiochem) in a final volume of 25 μl . Reactions were initiated by addition of $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ and terminated by spotting 10 μl of the reaction mixture onto P81 paper followed by washing in 1% phosphoric acid.

10 *Immunoprecipitation.* For immunoprecipitations, HEK293 cells in 35 mm dishes stably expressing CaMK-X1-X-press plasmid were washed twice in ice-cold PBS and lysed in solution containing 50 mM Tris/HCl, pH 7.6, 2 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol, protease inhibitors aprotinin (10 $\mu\text{g}/\text{ml}$) leupeptin (100 $\mu\text{g}/\text{ml}$) pepstatin (0.7 $\mu\text{g}/\text{ml}$), 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, and 1% Triton X-100 (Lysis buffer). Proteins were immunoprecipitated with the anti-X-press antiserum (1:100 dilution) or with control serum. The
15 immuno complexes were recovered using protein G Sepharose.

In vitro kinase assay with immunoprecipitated materials. CaMK-X1 was eluted from the immunocomplexes as described in the previous section and 20 μl of the eluate was mixed with 20 μl of phosphorylation mix containing 100 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (specific activity, 400-600 cpm/pmol), 30 mM Tris, pH 7.4, 30 mM MgCl_2 , 1 mM DTT, and 250 nM peptide and incubated for 10-15 minutes at 30°C .

20 *Northern Blot analysis.* Northern blot analysis was performed using an $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ -labeled CaMK-X1 cDNA fragment corresponding to bases 1.2 kb of human CaMK-X1 according to standard procedures (Ambion). RNA from several primary human tissues was analyzed with commercially available poly(A) + RNA blots (CLONTECH). The blotted membrane was dried and autoradiographed.

25 *CaMK-X1 activity assay.* Equivalent concentrations of purified CaMK-X1 preparations were incubated using a Beckman Biomek 2000 robotic system. Each well (96 well microtiter plate) contained 15 μl reaction mixture composed of 50 mM HEPES, pH 8.0, 10 mM MgCl_2 , 1 mM dithiothreitol, 0.005% Tween 20, 1 mM CaCl_2 , 1.5 mM Calmodulin (CalBiochem) 50 μM $\gamma\text{-}^{32}\text{P}$ ATP (200 cpm/pmol) and 0.2 $\mu\text{g}/\mu\text{l}$ Syntide 2 (American Peptide Company) or CREBtide (CalBiochem) in
30 a final volume of 25 μl . The reaction was initiated by addition of $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ and terminated by spotting 10 μl of the reaction mixture into a 96 well Millipore Multiscreen plate. The Multiscreen plate was washed in 1% phosphoric acid, dried and counted in a Wallac Microbeta scintillation counter.

Example 3

35

SGK2 α

The Genbank EST database was searched as described in Example 1. Analysis of the BLASTN and BLASTX outputs identified a EST sequence from IMAGE clone AF169034 that had potential for being associated with a sequence encoding a kinase domain-related protein, e.g., the sequence had homology, but not identity, to known kinase domain-related proteins.

The AF169034 IMAGE clone was sequenced using standard ABI dye-primer and dye-terminator chemistry on a 377 automatic DNA sequencer. Sequencing revealed that the sequence corresponds to SEQ ID NO:5, SGK2 α . The expression of SGK2 α was determined by dot blot analysis, and the protein was found to be upregulated in several tumor samples. SEQ ID NO:18 and 19 were used in amplification.

Dot blot preparation. Total RNA was purified from clinical cancer and control samples taken from the same patient. Samples were used from both liver and colon cancer samples. Using reverse transcriptase, cDNAs were synthesized from these RNAs. Radiolabeled cDNA was synthesized using Strip-EZTM kit (Ambion, Austin, TX) according to the manufacturer's instructions. These labeled, amplified cDNAs were then used as a probe, to hybridize to human protein kinase arrays comprising human SGK2 α . The amount of radiolabeled probe hybridized to each arrayed EST clone was detected using phosphorimaging.

The expression of SGK2 α was substantially upregulated in the tumor tissues that were tested. The data is shown in Table 4, expressed at the fold increase over the control non-tumor sample.

Table 4

	liver 1	liver 2	liver 3	colon 1	colon 4	colon 5	colon 7	colon 8	colon 9	colon 10
SGK2 α	3.6	2.4	1.1	1.1	1.0	3.9	1.8	1.4	0.7	2.55
beta-actin	2.05	1.07	1.57	0.42	1.28	2.19	1.20	4.60	0.60	0.49
GAPDH	1.30	0.33	1.25	0.76	Not done	Not done	Not done	Not done	Not done	Not done
K413 (ribosomal protein)	Not done	Not done	Not done	Not done	1.72	2.36	2.10	1.00	1.00	1.68

The data displayed in Table 5 provides a brief summary of the pathology report of the patient samples.

Table 5

Patient	Age	Gender	Precursor Adenoma	Site of Involvement	Differentiation	Vascular Invasion	Lymphatic Involvement	Metastasis
Liver 1	49	Female	N/a	Liver	Moderately Differentiated	No	Yes	No
Liver 2	53	Male	N/a	Liver	Moderately Differentiated	Yes	No	No
Liver 3	75	Female	Yes	Right Colon	Moderately differentiated	No	No	No
Colon 1	55	Female	No	Rectum	Moderately Differentiated	N/A	Yes	No
Colon 4	91	Female	Yes	Cecum	Moderately Differentiated	No	Yes	No
Colon 5	79	Male	No	Ileum and Colon	Moderately Differentiated	No	No	No
Colon 7	93	Male	No	Recto-Sigmoid	Moderately Differentiated	No	No	No
Colon	61	Male	Yes	Yes	Moderately	No	Yes	Yes,

8					Differentiated			Liver
Colon 9	60	Male	No	Recto-Sigmoid	Moderately Differentiated	Yes	No	Yes, Liver
Colon 10	60	Male	No	Sigmoid Colon	Moderately Differentiated	Yes	Yes	No

Creation of stable cell lines over expressing SGK2 in HEK293 cells. We constructed a mammalian expression vector encoding N-terminal Xpress tagged forms of the 45 kDa SGK2 kinase. The ORF of SGK2 was placed in frame with N-terminal Xpress and a Histidine tag in pcDNA 3 mammalian expression vector using standard PCR-based cloning techniques. To characterize SGK2 at the protein level, HEK293 cells were transfected and a stable cell line selected with pcDNA 3 His-Xpress-SGK2 plasmid in the presence of G418. HEK293 cells were stably transfected with mammalian vector incorporating SGK2 to produce clones over expressing wild type SGK2.

Briefly, cells were grown in d-MEM containing 5% FCS, 2mm L-glutamine, glucose (3.6 mg/ml) and G418 (40 µg/ml) was added to transfected cells to maintain selection pressure. The cell lysates were prepared from stable cell lines and subjected to Western blotting with anti-Xpress mAb and anti-His-antibody. A protein with a 45 kDa molecular mass was identified in lysates of HEK293 cells stably expressing SGK2. A similar protein could not be detected in the control HEK293 cells. This analysis suggests that HEK293 cells are overexpressing SGK2 as a fusion protein. To determine whether these cells express higher levels of SGK2 mRNA, we isolated mRNA from stable cell lines as well as control HEK293 cells. Equal amounts of mRNA were immobilized on a nylon membrane and subjected to hybridization with a specific SGK2 probe. Stable cell lines expressed a significantly higher concentration of SGK2 mRNA as compared to control HEK293 cells. These results indicate that stable cell lines are over expressing SGK2 mRNA as well as SGK2 protein. These stable cell lines were used in the subsequent experiments.

Overexpressed SGK2 can phosphorylate GSK3 in vivo. We explored the identification of the downstream effectors of SGK2 by using SGK2 overexpressing cells. SGKs have 54 % nucleotide sequence homology to PKB and it has previously been shown that PKB could phosphorylate GSK3 *in vivo* and *in vitro*. In view of this, we wanted to determine whether SGK2 could regulate the activity of GSK3, a kinase that is normally phosphorylates beta catenin. GSK3 phosphorylates beta catenin and targets it for destruction via a ubiquitin-proteasome pathway. To determine whether SGK2 could phosphorylate GSK3, initially, we carried out transient transfection assays in human embryonal kidney epithelial cells (HEK293). Transfection of SGK2 resulted in increased phosphorylation of GSK3. This was monitored by specific anti-GSK3 phospho Ser9 antibody. These results suggest that SGK2 effects the phosphorylation of GSK3 *in vivo*.

As a control, we measured the concentration of GSK3 protein in the assay. The concentration of GSK3 is not affected by SGK2 but the phosphorylation status of GSK3 is affected by the expression of SGK2. This is particularly significant at the lower concentration of serum (0.5%) and 0.1-0.2 µg concentration of SGK2 plasmid. Because GSK3 activity can be inhibited by phosphorylation, it is possible that inhibition of GSK3 by SGK2 could lead to other downstream

effects. To further evaluate the link between SGK2 and GSK3 we measured the phosphorylation status of GSK3 in HEK293 cells and in HEK293 cells stably transfected with SGK2 (named SGK-37A). SGK-37A cells overexpressing SGK2 had significantly higher phospho GSK3 than normal HEK293 cells.

5 This data demonstrates that SGK2 can modulate the phosphorylation status of GSK3 in stably transfected HEK293 cells. It has been shown that GSK3 phosphorylation leads to GSK3 inactivation (Cross *et al.* (1995) Nature 378:785-789). SGK2 may directly phosphorylate GSK3 and inactivate it, thereby abolishing phosphorylation of the cytoplasmic signaling molecule β -catenin and causing its stabilization and nuclear translocation. In the nucleus, β -catenin associates with TCF4 to
10 induce the transcription of several genes including cyclin D1.

SGK2 enhances cell proliferation. Since we have shown that overexpression of SGK2 stimulates GSK3 phosphorylation, it was investigated whether this could lead to cell proliferation. To study the effects of SGK2 on cell proliferation, we used several cells types. These cells were
15 transiently transfected with SGK2 or control DNA plasmids. The DNA synthesis rate was determined by measuring [³H] thymidine incorporation. When HEK293 and 3T3 cells were transfected with SGK2, they exhibited greater amounts of DNA synthesis than the control vector. The rate of proliferation was dependent on the concentration of transfected SGK2 plasmid. This data indicates that SGK2 stimulates cell proliferation in these cell types. Co-expression of PDK1 with SGK further
20 enhanced the rate of proliferation.

 These data reveal that SGK2 promotes proliferation in a variety of cells, and suggest that SGK2 promotes cell proliferation and support tumor progression in these types of cells.

SGK overexpression stimulates AP1 transactivation. It has previously been shown that
25 GSK3 phosphorylates c-Jun at C-terminal sites, resulting in inhibition of DNA binding (Nikolakaki *et al.* (1993) Oncogene 8:833-840) This can lead to the inhibition of AP1 activity in intact cells. It is believed that this keeps the cell's homeostasis in control. Since we have shown that SGK2 phosphorylates GSK3, we wanted to evaluate whether this could modulate the AP1 transactivation in cells overexpressing SGK2.

30 AP1 activity was measured in HEK293 cells and in HEK293 cells stably transfected with SGK2. SGK-37A clones have been shown to overexpress SGK2. AP1 activity was several fold higher in SGK-37A than in control HEK293 cells (Fig. 3). This data suggests that SGK2 can upregulate AP1 promoter activity in HEK293 cells. In the nucleus, AP1 transactivation induces the transcription of several genes involved in proliferation and several MMP genes. Our data suggests
35 that SGK2 can induce an invasive phenotype via AP1 dependent upregulation of MMP gene expression.

SGK2 stimulates the translocation of beta catenin into the nucleus. SGK2 stabilizes beta catenin in HEK293 cells. To determine whether overexpression of SGK2 in HEK293 cells would
40 induce beta catenin stability, we employed immunocytochemistry analysis. Monoclonal antibody for

beta catenin was used in the analysis. *In vivo* expression of beta catenin was measured by standard protocols. The results indicate that SGK2 expressing cells have a higher concentration of beta catenin than parental cells. β catenin is localized entirely in the nucleus of SGK2 overexpressing cells, suggesting that SGK2 regulates the translocation of beta catenin into the nucleus.

5 Taken together, these results indicate that SGK2 is an important intracellular regulator of signaling via components of the Wnt/wingless pathway, specifically through modulation of GSK3 β activity. Beta catenin has a consensus sequence phosphorylation site for GSK3 β , and GSK3 β acts to cause the degradation of beta catenin. Several studies have shown that GSK3 β phosphorylates β catenin and that the phosphorylation of β catenin is essential for its degradation. If β catenin is not
10 phosphorylated, the stability of β catenin increases in the cytoplasm and subsequently increases the translocation of beta catenin to the nucleus. In the nucleus, beta catenin associates with TCF4 to induce the transcription of several genes including cyclin D1.

SGK stimulates TCF4 transcriptional activity. The nuclear translocation of beta catenin is
15 associated with a complex formation between β catenin and members of the high mobility group transcription factors, LEF1/TCF, which then activate transcription of target genes. LEF1 is a transcription factor that is by itself unable to stimulate transcription from multimerized sites, although in association with β catenin LEF1/TCF proteins can augment promoter activity from multimerized binding sites.

20 We examined the transcriptional activation of a synthetic TCF4/ β catenin responsive promoter construct containing TCF4 binding sites in HEK293 cells overexpressing SGK2 and in control HEK293 cells. Higher promoter activity was observed only in SGK2 overexpressing cells. Transient transfection of increasing concentrations of TCF4 reporter gene produced concentration dependent TCF4 transactivation in SGK2 over expressing cells, whereas transient transfection of
25 TCF4 reporter gene into HEK293 cells did not produce significant transactivation. This result indicates that SGK2 selectively targets GSK3 β . Regulated β catenin subsequently increased the TCF4 transactivation in HEK293 cells. These data indicates that SGK2 overexpression overcomes the regulation of TCF4 expression by adhesion /deadhesion, and that it maintains constitutively high levels of TCF4 transactivation. TCF4/ β catenin has been shown to induce transcription of genes
30 encoding homeobox proteins that regulate mesenchymal genes, and this pathway is likely to mediate the epithelial to mesenchymal transformation. Constitutive activation of TCF/ β catenin is oncogenic in human colon carcinomas. The data presented here show that SGK2 can modulate β catenin signaling and transactivate TCF4 reporter genes.

35 *SGK2 stimulate NF- κ B transcription.* It has previously been shown that PKB/AKT regulate NF- κ B mediated transactivation. In view of this, we next asked whether SGK2 could activate the NF- κ B reporter assay *in vivo*. To evaluate NF- κ B transactivation, the NF- κ B promoter containing luciferase plasmid was transiently transfected into HEK293 cells overexpressing SGK2 and control HEK293 cells. As shown in Figure 3, the activity of the NF- κ B reporter was several fold higher in

SGK2 overexpressing cells than in control HEK293 cells. Increasing concentration of NF- κ B reporter plasmid in the SGK2 overexpressing cells increased luciferase activity, whereas NF- κ B mediated transactivation had no significant effect on the control HEK293 cell. This data demonstrates that SGK2 can regulate NF- κ B transactivation.

5 NF- κ B transactivation occurs in response to the major proapoptotic signals, including TNF- α , anticancer drugs, and ionizing radiation. Several reports have indicated that in some cancer cell types, NF- κ B is an important factor for cell survival. Hence, SGK2 may promote cell survival in certain cell types and participate in tumor promotion.

10 NF- κ B DNA binding activity coincides with degradation of I κ B alpha. To examine the status of I κ B alpha in the SGK2 overexpressing cells, we performed the following experiment. Cellular extracts were made from HEK293 cells overexpressing SGK2 and control HEK293 cells. These cell extracts were analyzed against a specific anti-phospho I κ B alpha antibody. Increasing concentrations of cell extract produced increasing I κ B alpha phospho signal, whereas the same protein concentration of control HEK293 cell extracts did not produce I κ B alpha phospho signals.
15 These results suggest that NF- κ B activation by SGK2 is mediated by I κ B alpha phosphorylation.

SGK2 phosphorylation of BAD. SGK2 phosphorylates some of the proteins phosphorylated by PKB. It has previously been shown that PKB can phosphorylate BAD. It was tested whether SGK2 phosphorylates BAD. Protein was isolated from HEK293 cells overexpressing SGK2 and
20 control HEK293 cells; and the phosphorylation status of BAD was measured. The cells were lysed and the expression of BAD phosphorylation was determined by anti-BAD phospho antibody. SGK2 overexpressing cells contain higher levels of phospho Bad protein than normal cells, although expression levels of BAD protein were unaffected by SGK2. These finding show that SGK2 increases BAD phosphorylation in HEK293 cells.

25 Phosphorylation of BAD may lead to the prevention of cell death via a mechanism that involves the selective association of phosphorylated forms of BAD with 14-3-3 protein isoforms. The identification of BAD as a SGK2 substrate expands the list of *in vivo* SGK2 targets. Recent studies have revealed that BAD represents a point of convergence of several different signal transduction pathways that are activated by survival factors that inhibit apoptosis in mammalian cells. These data
30 suggest that SGK2 inhibits apoptosis in mammalian cells through phosphorylation of BAD.

Phosphorylation of FKHR in HEK293 cells. The forkhead family of transcription factors is involved in tumorigenesis in rhabdomyosarcoma and acute leukemias. FKHR, FKHL1, and AFX mediate signaling via a pathway involving IGFR1, PI3K and PKB/AKT. Phosphorylation of FKHR
35 family members by PKB/AKT promotes cell survival and regulates FKHR nuclear translocation and target gene transcription. Insulin stimulation specifically promotes phosphorylation of this threonine site and causes FKHR cytoplasmic retention by 14-3-3 protein binding on the phosphorylated sequence.

To investigate whether FKHR could be phosphorylated by SGK2 in a cellular context, we created HEK293 cells stably expressing SGK2 and then examined FKHR phosphorylation with phospho specific antibodies. These experiments demonstrated that FKHR, Thr24 or Ser 256 were phosphorylated at low levels in normal HEK293 cells whereas HEK293 stable cells had higher levels of FKHR phosphorylation. This data shows that FKHR exhibits higher phosphorylation status in SGK2 overexpressing cells.

It has previously been shown that FKHR phosphorylation leads to FKHR's interaction with 14-3-3 proteins and sequestration in the cytoplasm, away from its transcriptional targets. The unphosphorylated FKHR accumulates in the nucleus where it activates death genes, including Fas ligand gene, and thereby participates in the process of apoptosis. Upon phosphorylation, FKHR interacts with 14-3-3 and is retained in the cytoplasm thereby inhibiting its ability to activate transcription. Therefore, phosphorylation of FKHR by SGK2 can promote cell survival.

CREB phosphorylation is regulated by SGK2. To determine whether CREB is a regulatory target for SGK2, we performed the following experiments. Equal amounts of protein were isolated from SGK2 overexpressing cells as well as control HEK293 cells and subjected to phospho CREB analysis. The cells were lysed and the amount of CREB phosphorylation was determined by CREB phospho (Ser133) antibody. SGK2 overexpressing cells contain higher levels of phospho CREB protein than normal cells, showing that SGK2 increases CREB phosphorylation

Studies by have indicated that CREB function is important in promoting cell survival. Cyclin D1 expression is regulated by CREB. The majority of breast cancer cell lines and mammary tumors overexpress cyclin D1, suggesting that induction of cyclin D1 may play an important role in mammary tumorigenesis. These studies further clarify the mechanism by which SGK2 could promote cell survival. CREB function is important in promoting cell survival by responding to growth factor stimulation. These data imply that SGK2 modulates the phosphorylation status of CREB *in vivo*, and therefore is involved in cell survival through the CREB pathway.

SGK2 is activated by PDK1 and the activation leads to increased kinase activity. To determine whether cloned and purified SGK2 can phosphorylate specific peptides directly, SGK2 was purified from insect cells. Activation was performed *in vitro* by mixing SGK2 and PDK1. After the activation, the PDK1 was removed from the mixture and purified SGK2 was used for the analysis. The cell extracts were purified by GST affinity column chromatography and the purity was analyzed by SDS- PAGE. Both non-activated and PDK1-activated SGK2 produced similar amounts of protein. SGK2 activated by PKD1 was significantly phosphorylated, while non-activated SGK2 was not. The data is shown in Figure 4.

The kinase activity of SGK2 was evaluated using specific peptides. SGK2 was incubated with two different peptide substrates ((SEQ ID NO: 32) PKB -sub: CKRPRAASFAE; and (SEQ ID NO:33) PDK1: KTF CGTPEYLAPEV RREPRILS EEEQEMFRDFDYI (UBI Catalogue #12401), and *in vitro* kinase assays carried out. Equivalent concentration of purified SGK2 were incubated using a Beckman Biomek 2000 robotic system. Each well containing 25 μ l reaction mixture composed of 10

5 μ l SGK2, 5 μ l of assay dilution buffer, 5 μ l of peptide substrate and 5 μ l of γ 32 P-ATP. The kinase reaction was carried out for 15 minutes at room temperature (22°C). At the end of the reaction period, 10 μ l of the reaction mixture was spotted onto 96-well p81 phosphocellulose multiscreen plates from Millipore, washed and the 32 P incorporation was counted in a Wallac Microbeta scintillation counter.

Peptides incubated with purified SGK 2 gave significant incorporation of 32 P, whereas in the absence of peptides no significant incorporation was seen. When comparing the peptides, PKB-sub had significant incorporation of 32 P whereas addition of same amount of control peptide (PDK1 peptide) had no significant incorporation. This data demonstrates that purified SGK2 possesses a kinase activity *in vitro*. Moreover, the PDK1 activated SGK2 had significantly higher kinase activity compared to non-activated SGK2. These data clearly demonstrate that activated SGK2 phosphorylates the GSK3 Ser9 (GSK3 β consensus) sequence, supporting the previous observation that SGK2 overexpressing cells exhibit higher level of GSK3 Ser9 phosphorylation than control cells.

15 *SGK2 kinase activity is stimulated by Calyculin A and Okadaic acid.* Hi5 insect cells expressing GST-SGK2 were treated with 100 nM microcysteine, 99.8 nM okadaic acid and 49.8 nM calyculin A for four hours at 27°C. The GST-SGK2a fusion protein was purified by GST-agarose affinity column and eluted with 20mM Glutathione/50mM Tris-HCl/50mM NaCl, pH 7.5. Substrates were PKB sub and CapK sub at 1mg/ml, for 15 minutes at room temperature. The results were as follows:

	No-Substrate (CCPM1)	PKB sub (CCPM1)	CapK sub(CCPM1)
Untreated	349	979	1081
Microcysteine	305	217	330
Calyculin A	0	92540	59335
Okadaic Acid	2078	132171	161553

These data indicate that okadaic acid and Calyculin A stimulated SGK2 kinase activity, suggesting that okadaic and Calyculin A can stimulate SGK2 activity. It has previously been shown that protein phosphatase inhibitors such as okadaic acid and Calyculin A modulate phosphorylation of several nuclear proteins.

These findings demonstrate SGK2 could promote cell survival and cell growth. Overexpression of SGK2 in HEK293 cells increased GSK3 phosphorylation thereby inhibiting the activity of GSK3, and subsequently leading to AP1 transactivation. GSK3 is involved in regulation of several intracellular signaling pathways, of which the Wnt pathway is of particular interest. In mammals, Wnt signaling increases the stability of beta catenin resulting in transcriptional activation of LEF-1/TCF. In SGK2 overexpressing cells we have shown increased LEF-1/TCF transactivation through increasing the stability of the beta catenin pool in the cells, suggesting that SGK2 activates

the Wnt signaling pathway, which can lead to nuclear localization of beta catenin and increased transactivation of LEF-1/TCF.

At least 6 SGK2 substrates have been identified in mammalian cells, and they fall into two main classes: regulators of apoptosis and regulators of cell growth, including protein synthesis and glycogen metabolism. The SGK2 substrates involved in cell/death regulation include Forkhead transcription factors (FKHR), the pro-apoptotic Bcl-2 family member BAD, and the cyclic AMP response element binding protein (CREB).

We have also demonstrated that SGK2 could regulate signaling pathways that lead to induction of the NF- κ B family of transcription factors in HEK293 cells. This induction occurs at the level of degradation of the NF- κ B inhibitor I κ B and is specific for NF- κ B. These data suggest that SGK2 appears to be a point of convergence for several different signaling pathways. Taken together, our results suggest that the over expression of SGK2 may therefore play a central role in tumor cell progression.

Materials and Methods.

Buffers, reagents and methods were as described in Example 2, unless otherwise stated.

Cloning of full length SGK2. To generate the full length cDNA of SGK2, a pair of primers were designed and used in a PCR reaction. The amplification product was cloned through restriction sites, EcoR I and Xho I, into bacteria expression vector pGEX-4T-3 and mammalian expression vector pcDNA3.1/His B. All constructs were verified by restriction enzyme digestion and DNA sequencing.

Expression and Purification of SGK2 Protein. The human SGK2 gene was subcloned into baculovirus transfer vector pAcG2T (BD Pharmingen) under the control of the strong AcNPV (*Autographa californica* Nuclear Polyhedrosis Virus) polyhedrin promoter. This was co-transfected with linear BaculoGoldTM DNA in *Spodoptera frugiperda* Sf9 cells following the manufacturer's procedure (BD Pharmingen). The high titer of GST-SGK2 recombinant baculovirus was amplified in Sf9 cells in TNM-FH medium (JHR Biosciences) with 10% fetal bovine serum. The GST-SGK2 protein was expressed in about 5×10^8 Hi5 cells (Invitrogen) in 500 ml of Excell-400 medium (JHR Biosciences) with about 5 MOI for a period of 72 h in a spinner flask. The cells were harvested at 800Xg for 5 min at 4°C. The pellet was lysed in 40 ml of Lysis Buffer by sonication and centrifuged at 10,000Xg at 4°C for 15 min. The supernatant was loaded on the column contained 2.5 ml of glutathione-agarose (Sigma). The column was washed with Wash Buffer A until OD280 returned to baseline, then Wash Buffer B. The GST-SGK2 protein was eluted in Elution Buffer. The fraction was aliquoted and stored at -70°C.

Assay of SGK2. SGK2 was assayed at room temperature for 15 min with 25 μ l of reaction mixture containing 5 mM MOPS, pH 7.2, 5 mM MgCl₂, 5 mM β -glycerophosphate, 50 μ M dithiothreitol, 1 μ M β -methyl aspartic acid, 1 mM EGTA, 0.5 mM EDTA, 0.5 μ M PKI, 50 μ M [γ -³²P]-ATP and 0.2 μ g/ μ l PKB-sub peptide (UBI) or PDKtide peptide (UBI) as substrates. GSK3 consensus

peptide (SEQ ID NO:34, PKB -sub: CKRPRAASFAE), PDK1 sub- SEQ ID NO:35, KTFCGTPEYLAPEVRREPRILSEEEQEMFRDFDYI. Reactions were initiated by addition of [γ - 32 P]-ATP and terminated by spotting 10 μ l of aliquots onto cellulose phosphate paper in 96-well filtration plate (Millipore), followed by washing in 1% phosphoric acid. The dried plate was added 25 μ l scintillant (Amersham) and counted.

SGK2 Phosphorylation by PDK1. SGK2 was incubated with active His-tag PDK1 in the presence of Mg^{2+} /ATP. His-tag PDK1 was expressed in insect cells and purified on Talon affinity column. SGK2 phosphorylation assay was performed at room temperature for 20 min in 25 μ l of reaction solution consisting of 10 mM MOPS, PH 7.2, 15 mM $MgCl_2$, 5 mM β -glycerophosphate, 1 mM EGTA, 0.2 mM sodium orthovanadate, 0.2 mM dithiothreitol, 0.5 μ M PKI, 0.2 μ M Microcystin-LR, 75 ng/ μ l PtdIns (3,4,5) P3 (PIP3), 156 ng/ μ l dioleoyl phosphatidylcholine (DOPC), 156 ng/ μ l dioleoyl phosphatidylserine (DOPS), 50 μ M [γ - 32 P]-ATP, ~20 ng His-PDK1 and ~5 μ g GST-SGK2. The reaction were incubated and terminated by addition of 25 μ l 2X loading buffer. No PDK1 was added to negative control reaction. 25 μ l of above loading samples were run on 9% SDS-PAGE. The dried Coomassia blue-stained gel was imaged in GS-525 Molecular Imager System (BIO-RAD).

SGK2 Activation by PDK1. About 2.5 mg of GST-SGK2 and 1 μ g of His-PDK1 were incubated at 4°C for 2 hours in 20 ml of activation solution containing 10 mM MOPS, PH 7.2, 15 mM $MgCl_2$, 5 mM β -glycerophosphate, 1 mM EGTA, 0.2 mM sodium orthovanadate, 0.2 mM dithiothreitol, 0.5 μ M PKI, 0.2 μ M Microcystin-LR, 75 ng/ μ l PtdIns (3,4,5) P3 (PIP3), 156 ng/ μ l dioleoyl phosphatidylcholine (DOPC), 156 ng/ μ l dioleoyl phosphatidylserine (DOPS), and 10 mM ATP. The glutathione was removed from the activation solution on Q-sepharose column. The activated GST-SGK2 were re-purified from glutathione-agarose column.

Cell and cell culture. 293 cells were stably transfected with a mammalian vector incorporating SGK2 to produce overexpressing wild type SGK2. Cells were grown in MEM containing 10 % FCS, 2 mM L-glutamine, glucose (3.6 mg/ml), insulin (10 μ g/ml), and G418 (40 μ g/ml) were added to transfected cells to maintain selection pressure.

Transient transfection: HEK293 cells were seeded at 1.5×10^5 cells/well plate and grown for 24 hr before transfection. Various concentration of plasmid DNA were transfected using Eugene (Roche) according to the manufacture's protocol. DNA content was normalized with appropriate empty expression vectors. Cells were starved for O/N in DMEM containing 0.5 % FBS.

Western blotting: Cells were lysed for 10 minutes on ice in NP-40 lysis buffer (1% NP40, 50 mM Hepes, pH 7.4, 150 mM NaCl, 2mM EDTD, 2mM PMSF, 1mM Na-o- vanadate, 1 mM NaF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin). Extracts were centrifuged with the resulting supernatants being the cell lysate used in assays. Lysates were electrophoresed through SDS-PAGE and transferred to Immobilon-P (Millipore Bedford, MD). Antibodies used to probe Western blots were: Anti-Xpresss, Phospho-FKHR (Thr24, Caspase-9, Phospho-IkBalpha (Ser32/36), Bad, Phospho CREB, Phospho GSK3 alpha (ser-9), GSK3 monoclonal, (New England Biolab, Mississauga, ON, Canada) Bands were visualized with ECL chemiluminescent substrate (Amersham Pharmacia biotech).

Reporter assay: 293 cells were transfected in 6-well plates with Eugene (Roche Diagnostics) according to the manufacture's instructions. To analyse various reporter assay, respective reporter construct were transiently transfected with indicated amount of luciferase reporter gene construct series of LEF-1/TCF binding sites, AP1 binding sites and NF- κ B binding sites. Extracts were prepared and assayed 24-48 after transfection and relative luciferase activity was determined using Promega Dual luciferase reporter assay system as described by the manufacture.

Immunocytochemistry: 293-cell lines were grown in 8 chamber slides for 2 days, washed with PBS, fixed in absolute cold methanol for 10 minutes, washed with PBS and incubated overnight at 4° C with beta-catenin (#C19220-BD Transduction Laboratories), His -Prob (#Sc-803, Santa Cruz, USA) and anti-Xpress antibody (R910-25, Invitrogen), all diluted 1:100 in PBS with 0.1 % Triton X-100, then washed with PBS. Proceed with immunostaining by using the ABC method (ABC-Elite kit, Vector). According to the amount and intensity of staining, the scale was divided into 2 classes. The slides designated "+" had positive staining intensity, slides designated "-" showed no immunoreactivity. In addition to conventional light microscopic examination, in order to quantitate the amount of reactivity, specimens were also investigated by computerized image analysis using Image pro (Media Cybernetics, MD, USA).

Expression and Purification of GST-SGK2a from Hi 5 Insect cells. Human SGK2a was cloned into the Baculovirus vector pAcG2T with the multiple cloning sites in the vector.. This vector contains an N-terminal Glutathione S-transferase tag (GST-tag) which allows for affinity purification on Glutathione agarose beads. The vector was infected into Sf9 insect cells via lipid vesicles. The titer of the baculovirus particles was amplified in Sf9 insect cells. The amplified baculovirus titer was then used to infect four 250 ml volume spinner-flasks (Pyrex) containing Hi 5 cells which were at approximately 0.8×10^6 cells/ml. The expression of the fusion protein cells were incubated at 27°C, with spinning at 80 rpm, over 3.5 days. Near the end of this expression period, each of the four 180 ml cultures of Hi 5 cells were stimulated with a 4 hour, 27° C treatment with either 100% DMSO (negative control) or one of three different PP1 and PP2a phosphatase inhibitors: 100 nM Microcystin (Calbiochem), 55.05 nM Calyculin A (Calbiochem), and 96.9 nM Okadaic Acid (Calbiochem). Finally, the cells were collected by centrifugation in Beckman Avant-25 rotor ID 10.500 at 3000 rpm, 5 min, 4°C. After a brief 1xPBS wash, the cells were resuspended in a 50 mM Tris-HCl / 1% NP-40, pH 7.5 lysis buffer supplemented with the following protease inhibitors: 100 μ M Sodium Vanadate, 1 mM glycerophosphate, and 237 μ l Protease Inhibitor Cocktail Set III (Calbiochem). The cells were lysed using the small probe of the sonic dismembrator: output 1:3 repetitions of 8 sec on and 5 sec pause. Once the cytosolic proteins are released into the supernatant, the cellular debris is removed by centrifugation in Beckman Avanti-30: 14,000 rpm, 15 min, 4°C. The lysate supernatant is applied to Glutathione-agarose beads (SIGMA) and allowed to batch-bind, rotating end-over-end, at 4°C for 30 mins. Non-specific proteins are washed from the beads 5 times with STEL 500 (50 mM Tris-HCl / 500 mM NaCl, pH 7.5) followed by 5 times with STEL 50 (50 mM Tris-HCl / 50 mM NaCl, pH 7.5). Finally, the GST-tagged fusion protein is eluted from the beads with Elution buffer (20 mM glutathione / 50 mM Tris-HCl / 50 mM NaCl). Purified SGK2a kinase activity is assayed against PKB

peptide SEQ ID NO:36 (CKRPRAASFAE), a universal SRC kinase family substrate and CapK peptide SEQ ID NO:37 (CGRTGRRNSI).

Example 4

GRK5

Genbank sequences were screened as described in Example 1. Analysis of BLASTN and BLASTX outputs identified a EST sequence from IMAGE clone A1358974 that had potential for being associated with a sequence encoding a kinase domain-related protein, e.g., the sequence had homology, but not identity, to known kinase domain-related proteins.

The A1358974 IMAGE clone was sequenced using standard ABI dye-primer and dye-terminator chemistry on a 377 automatic DNA sequencer. Sequencing revealed that the sequence corresponds to SEQ ID NO:7. SEQ ID NO:20 and 21 were used for amplification.

The expression of GRK5 was determined dot blot analysis, and the protein was found to be upregulated in several tumor samples.

Dot blot preparation. Total RNA was purified from clinical cancer and control samples taken from the same patient. Samples were used from both liver and colon cancer samples. Using reverse transcriptase, cDNAs were synthesized from these RNAs. Radiolabeled cDNA was synthesized using Strip-EZ™ kit (Ambion, Austin, TX) according to the manufacturer's instructions. These labeled, amplified cDNAs were then used as a probe, to hybridize to human protein kinase arrays comprising human GRK5. The amount of radiolabeled probe hybridized to each arrayed EST clone was detected using phosphorimaging.

The expression of GRK5 was substantially upregulated in the tumor tissues that were tested. The data is shown in Table 6, expressed at the fold increase over the control non-tumor sample.

Table 6

	liver 1	liver 2	liver 3	colon 1	colon 4	colon 5	colon 7	colon 8	colon 9	colon 10
GRK5	1.5	0.7	2.6	1.8	1.3	4.3	1.9	0.4	0.7	2.00
beta-actin	2.05	1.07	1.57	0.42	1.28	2.19	1.20	4.60	0.60	0.49
GAPDH	1.30	0.33	1.25	0.76	Not done	Not done	Not done	Not done	Not done	Not done
K413 (ribosomal protein)	Not done	Not done	Not Done	Not Done	1.72	2.36	2.10	1.00	1.00	1.68

Expression of GRK5. To characterize GRK5 at the protein level, Hi5 cells were transfected with pAcG4T3-GRK5. The ORF was cloned into baculovirus expression vector pAcG2T (BD pharmagen). This construct construct was then co-transfected with linear BaculoGold DNA into Sf9 cells to obtain an isolated recombinant virus. The recombinant virus was amplified and then used to infect sf9 cells. GRK5 expressed in Hi5 cells was purified by glutathione-sepharose column chromatography. Cell lysates were prepared from these cell lines for further analysis. Briefly, the precipitations were performed with ectopically expressed tagged GRK5 from insects cells as described in the method section. This will enable us to perform *in vitro* kinase assays for the identification of specific inhibitors of this kinase.

To characterize GRK5 at the protein level, HEK293 cells were transfected with pcDNA3-X-press-GRK5 by standard methods. The transiently transfected cell lines were used to prepare whole cell lysates which were analysed by Western blotting with an anti-X-press monoclonal antibody. These experiments revealed a fusion protein in the stably transfected cell lines, whereas HEK293 cell lines transfected with the vector only control did not have this protein. Similarly, we also detected GRK5 in transfected Hi5 cells.

The anti-X-press antibody was used to purify the kinase via immunoprecipitation. Anti-X-press antibody precipitated fusion protein was subjected to SDS-PAGE analysis. SDS-PAGE indicated that we could successfully purify the GRK5 from the lysates from transfected cells.

Next, anti-X-press antibody immunoprecipitated materials and glutathione-sepharose chromatography purified materials were used for *in vitro* kinase assays. Casein, MBP and phosphatidylserine were found to be phosphorylated by purified GRK5. In the absence of substrate there was no significant incorporation of radioactive materials (^{32}P) indicating that GRK5 does not autophosphorylate under these conditions. This suggests that glutathione-sepharose and X-press antibody purified materials possess a kinase activity and that this kinase activity is capable of phosphorylating substrates *in vitro*.

Expression and Purification of GRK5 Protein. The human GRK5 gene was subcloned into baculovirus transfer vector pAcG4T3 derived from pAcG2T (BD Biosciences) under the control of the strong AcNPV (*Autographa californica* Nuclear Polyhedrosis Virus) polyhedrin promoter. This was co-transfected with linear BaculoGold DNA in *Spodoptera frugiperda* Sf9 cells using standard techniques (BD Biosciences). The GST-GRK5 recombinant baculovirus was amplified in Sf9 cells in TNM-FH medium (JHR Biosciences) with 10% fetal bovine serum. The GST-GRK5 protein was expressed in about 5×10^8 Hi5 cells (Invitrogen) in 500 ml of Excell-400 medium (JHR Biosciences) at a multiplicity of infection (MOI) of five for 72 h in a spinner flask. The cells were harvested at 800Xg for 5 min at 4°C. The pellet was lysed in 40 ml of Lysis Buffer by sonication and centrifuged at 10,000Xg at 4°C for 15 min. The supernatant was loaded onto a column containing 2.5 ml of glutathione-sepharose (Sigma). The column was washed with Wash Buffer A until OD280 returned to baseline. The column was then washed with Wash Buffer B. The GST-GRK5 protein was eluted in Elution Buffer. The eluted protein was aliquoted and stored at -70°C.

Example 5

DM-PK

The Genbank EST database was searched as described in Example 1. Analysis of the BLASTN and BLASTX outputs identified a EST sequence from IMAGE clone A1886007 that had potential for being associated with a sequence encoding a kinase domain-related protein, e.g., the sequence had homology, but not identity, to known kinase domain-related proteins. The A1886007 IMAGE clone was sequenced using standard ABI dye-primer and dye-terminator chemistry on a 377 automatic DNA sequencer. Sequencing revealed that the sequence corresponds to SEQ ID NO:9. SEQ ID NO:22 and 23 were used for amplification. The expression of DM-PK was determined dot

blot analysis, and the protein was found to be upregulated in several tumor samples. As shown in Figure 5, a number of isoforms of DMPK were characterized, including SEQ ID NO:10; SEQ ID NO:38 and SEQ ID NO:39.

- 5 *Dot blot preparation.* Total RNA was purified from clinical cancer and control samples taken from the same patient. Samples were used from both liver and colon cancer samples. Using reverse transcriptase, cDNAs were synthesized from these RNAs. Radiolabeled cDNA was synthesized using Strip-EZTM kit (Ambion, Austin, TX) according to the manufacturer's instructions. These labeled, amplified cDNAs were then used as a probe, to hybridize to human protein kinase
- 10 arrays comprising human DM-PK. The amount of radiolabeled probe hybridized to each arrayed EST clone was detected using phosphorimaging.

The expression of DM-PK was substantially upregulated in the tumor tissues that were tested. The data is shown in Table 7, expressed at the fold increase over the control non-tumor sample.

15

Table 7

	liver 1	liver 2	liver 3	colon 1	colon 4	colon 5	colon 7	colon 8	colon 9	colon 10
DM-PK	1.8	1.2	2.8	2	2.0	1.7	4.5	0.9	1.2	2.35
beta-actin	2.05	1.07	1.57	0.42	1.28	2.19	1.20	4.60	0.60	0.49
GAPDH	1.30	0.33	1.25	0.76	Not done	Not done	Not done	Not done	Not done	Not done
K413 (ribosomal protein)	Not done	Not done	Not done	Not done	1.72	2.36	2.10	1.00	1.00	1.68

The data displayed in Table 8 provides a brief summary of the pathology report of the patient samples.

Table 8

Patient	Age	Gender	Precu- sor Adeno- ma	Site of Involve- ment	Differentiation	Vascular Invasion	Lymphatic Involvement	Metastasis
Liver 1	49	Femal e	N/a	Liver	Moderately Differentiated	No	Yes	No
Liver 2	53	Male	N/a	Liver	Moderately Differentiated	Yes	No	No
Liver 3	75	Femal e	Yes	Right Colon	Moderately differentiated	No	No	No
Colon 1	55	Femal e	No	Rectum	Moderately Differentiated	N/A	Yes	No
Colon 4	91	Femal e	Yes	Cecum	Moderately Differentiated	No	Yes	No
Colon 5	79	Male	No	Ileum and Colon	Moderately Differentiated	No	No	No
Colon 7	93	Male	No	Rectosi gmoid	Moderately Differentiated	No	No	No
Colon 8	61	Male	Yes	Yes	Moderately Differentiated	No	Yes	Yes, Liver

Colon 9	60	Male	No	Recto- Sigmoid	Moderately Differentiated	Yes	No	Yes, Liver
Colon 10	60	Male	No	Sigmoid Colon	Moderately Differentiated	Yes	Yes	No

5 *Expression of DM-PK in E. coli.* To characterize DM-PK at the protein level, *E. coli* cells were transformed with pGEX-DM-PK. The DM-PK ORF was cloned into a pGEX vector (Pharmacia) that was used to transform *E. coli*. A transformed colony was selected and cultured in order to express the GST-DM-PK fusion protein. The fusion protein was purified via glutathione-sepharose column chromatography. The purified fraction was analysed by SDS-PAGE, and showed a band corresponding to the DM-PK protein.

10 As an alternative expression system, we transfected HEK293 cells with DM-PK. Cell lysates of the transfected cells were prepared. We utilized an anti-X-press antibody to immunoprecipitate the recombinant DM-PK. This data shows successful expression and purification of DM-PK from transfected HEK293 cells.

15 *Kinase Activity.* DM-PK purified from both *E. coli* and transfected HEK293 was used for *in vitro* kinase assays. MBP and Histone H1 were both phosphorylated by purified DM-PK in these assays. In the absence of added substrate, there was no significant incorporation of radioactive materials (^{32}P) indicating that DM-PK does not autophosphorylate under these conditions. This data shows that purified DM-PK possesses kinase activity.

20 *Experimental procedures.* DM-PK was subcloned into bacterial expression vector pGEX-4T3 (Pharmacia) using EcoR1 and Not I sites. The GST-DM-PK protein was produced in *E. coli* DH5a cells in 2X YT media in 150 μM IPTG at 37°C overnight. The cells were harvested at 10,000Xg for 10 minutes at 4°C. The pellet was suspended in 50 ml of Lysis Buffer (150 mM Tris-HCl pH 7.5, 2.5 mM EDTA, 150 mM Mg Cl₂, 1% NP-40, 0.1 % β -mercaptoethanol, 0.1 mM PMSF, 1mM benzamide and 10 $\mu\text{g/ml}$ trypsin inhibitor), sonicated, and centrifuged at 10,000Xg for 15 minutes at 4°C. The supernatant was loaded onto a 3 ml glutathione-sepharose column. The column was washed by wash buffer (50 mM Tris-HCl, pH 7.5, 1mM EDTA, 500 mM NaCl, 0.1% β -mercaptoethanol, 0.1% NP-40, 0.1 mM PMSF and 1 mM benzamide) and eluted with standard elution buffer.

Example 6

PDK2 Sequence

30 The Genbank database was searched for ESTs showing similarity to known kinase domain-related proteins as described in Example 1. Analysis of the BLASTN and BLASTX outputs identified a EST sequence from IMAGE clone Af309082 that had potential for being associated with a sequence encoding a kinase domain-related protein, *e.g.*, the sequence had homology, but not identity, to known kinase domain-related proteins. The Af309082 IMAGE clone was sequenced using standard ABI dye-primer and dye-terminator chemistry on a 377 automatic DNA sequencer. Sequencing revealed that the sequence corresponds to SEQ ID NO:11; and a second sequence corresponds to SEQ ID NO:13.

Total RNA was purified from clinical cancer and control samples, and cDNAs synthesized by reverse transcriptase. CDNA corresponding to normal and tumor tissue from the same set were simultaneously amplified and labeled with alpha dCTP. Labeled, amplified cDNAs were then used to hybridize to human protein kinase arrays containing 354 protein kinases. The amount of radiolabeled probe hybridizing to each arrayed EST clone was detected using phosphorimaging. Through this process it was determined the PDK2 was upregulated in both colon and liver tumor tissue as compared to matched control tissue.

WHAT IS CLAIMED IS:

1. A method of screening for biologically active agents that modulate a cancer associated protein kinase function, the method comprising: combining a candidate biologically active agent with any one of:

5 (a) a polypeptide encoded by any one of SEQ ID NO:1, 3, 5, 7, 9, 11 or 13; or having the amino acid sequence set forth in SEQ ID NO:38 or SEQ ID NO:39;

(b) a cell comprising a nucleic acid encoding a polypeptide encoded by any one of SEQ ID NO:1, 3, 5, 7, 9, 11 or 13; or

10 (c) a non-human transgenic animal model for cancer associated kinase gene function comprising one of: (i) a knockout of a gene corresponding any one of SEQ ID NO:1, 3, 5, 7, 9, 11 or 13; (ii) an exogenous and stably transmitted mammalian gene sequence comprising polypeptide encoded by any one of SEQ ID NO:1, 3, 5, 7, 9, 11 or 13; and
determining the effect of said agent on kinase function.

15 2. A method for the diagnosis of cancer, the method comprising:
determining the upregulation of expression in any one of SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 38 or 39 in said cancer.

20 3. The method of Claim 2, wherein said cancer is a liver cancer.

4. The method of Claim 2, wherein said cancer is a colon cancer.

25 5. The method of Claim 2, wherein said determining comprises detecting the presence of increased amounts of mRNA in said cancer.

6. The method of Claim 2, wherein said determining comprises detecting the presence of increased amounts of protein in said cancer.

30 7. A method for inhibiting the growth of a cancer cell, the method comprising downregulating activity of the polypeptide encoded by any one of SEQ ID NO:1, 3, 5, 7, 9, 11 or 13 or having the amino acid sequence set forth in SEQ ID NO:38 or SEQ ID NO:39; in said cancer cell.

35 8. The method according to Claim 7, wherein said method comprises introducing antisense sequences specific for any one of SEQ ID NO:1, 3, 5, 7, 9, 11 or 13.

9. The method according to Claim 7, wherein said method comprises introducing an inhibitor of kinase activity into said cancer cell.

40 10. The method according to Claim 7, wherein said cancer cell is a liver cancer cell.

11. The method according to Claim 7, wherein said cancer cell is a colon cancer cell.

12. A method of screening for targets of a cancer associated protein kinase, wherein said targets are associated with signal transduction in cancer cells, the method comprising:

5 comparing the pattern of gene expression in a normal cell, and in a tumor cell characterized by up-regulation of any one of SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 38 or 39.

13. The method according to Claim 12, wherein said comparing the pattern of gene expression comprises quantitating specific mRNAs by hybridization to an array of polynucleotide probes.

10

14. A method of screening for targets of a cancer associated protein kinase, wherein said targets are associated with signal transduction in cancer cells, the method comprising:

15 comparing the pattern of protein phosphorylation in a normal cell, and in a tumor cell characterized by up-regulation of any one of SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 38 or 39.

15. The method according to claim 12 or claim 14, wherein said signal transduction involves activation by protein dependent kinase 1.

20 16. An isolated nucleic acid comprising the sequence set forth in any one of SEQ ID NO:1, 3, 5, 7, 9, 11 or 13.

Proliferation Assay on Cell Lines Transfected with K283

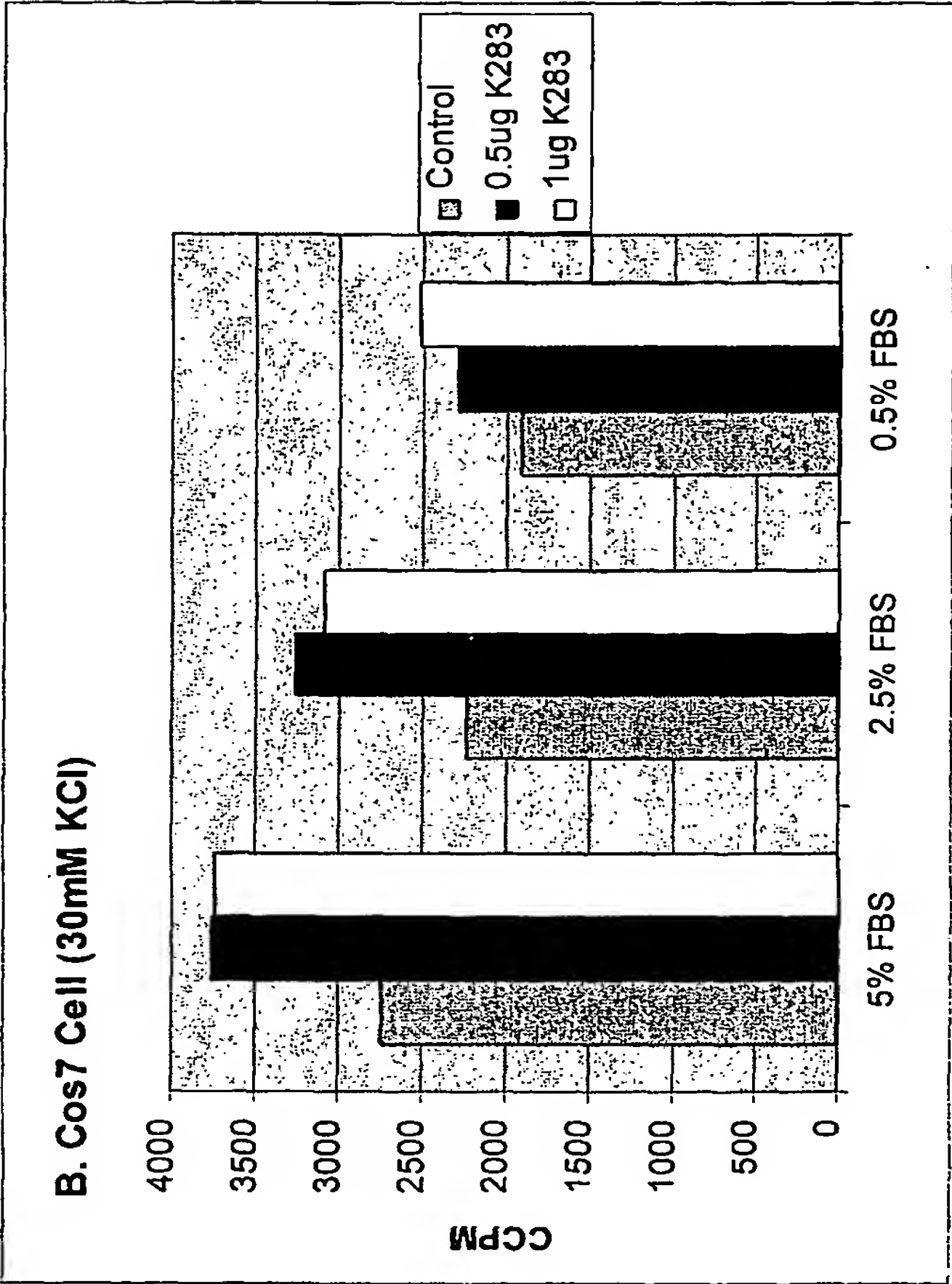
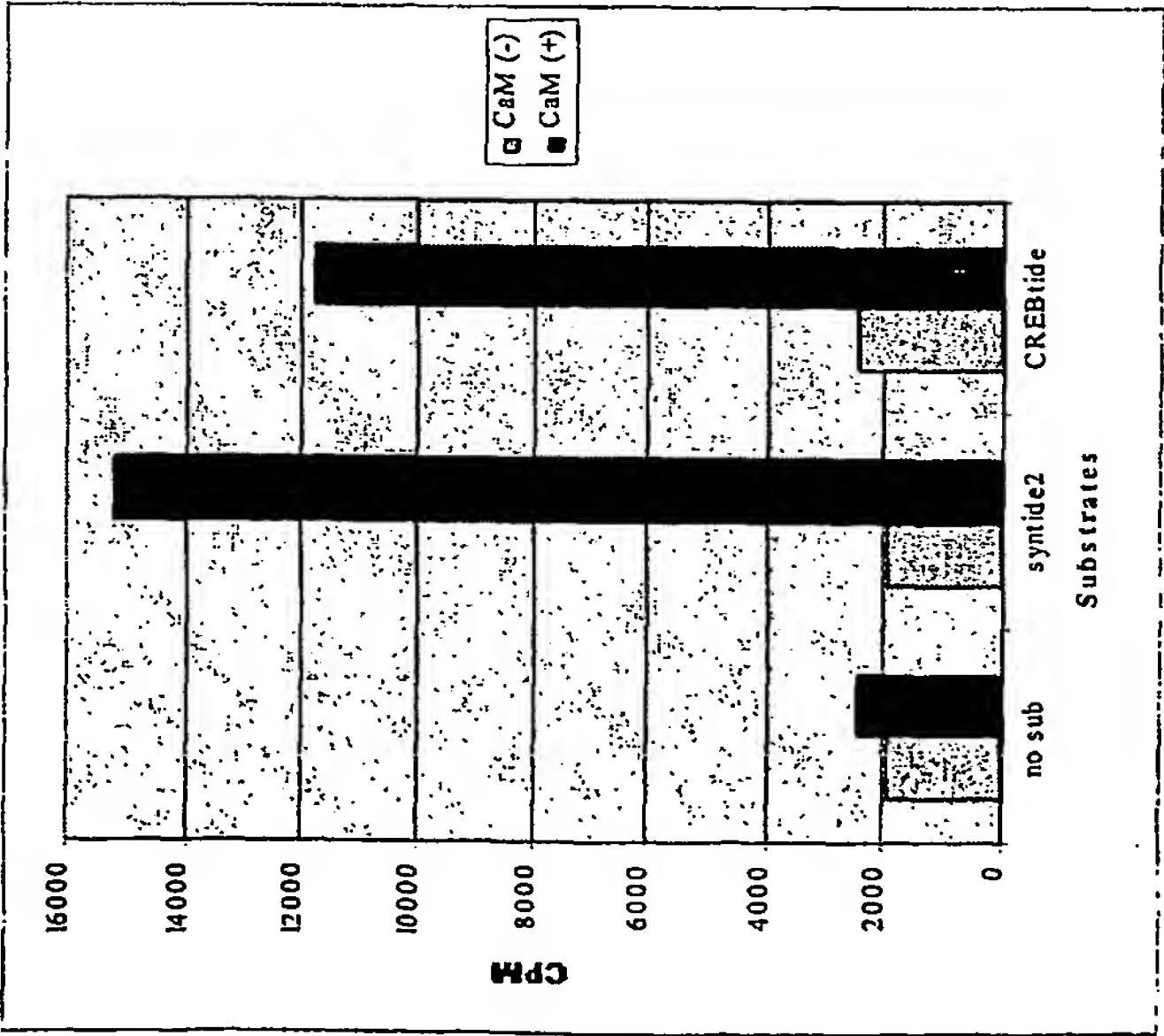


FIGURE 1

Kinase Activities of Expressed CaMKXI

A. CaMKXI Expressed in Hi5 Cell



B. CaMKXI Expressed in 293 Cell

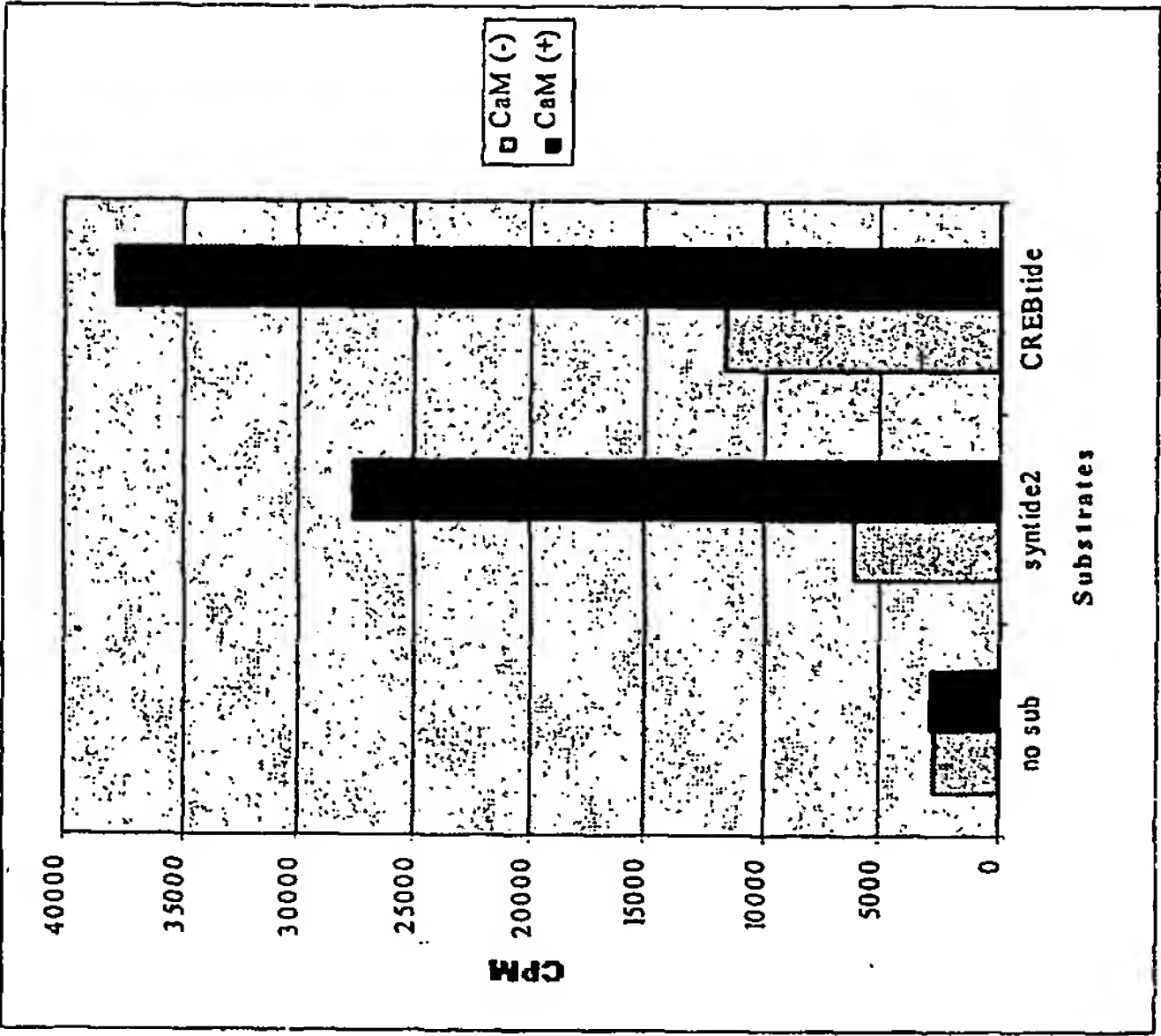


FIGURE 2

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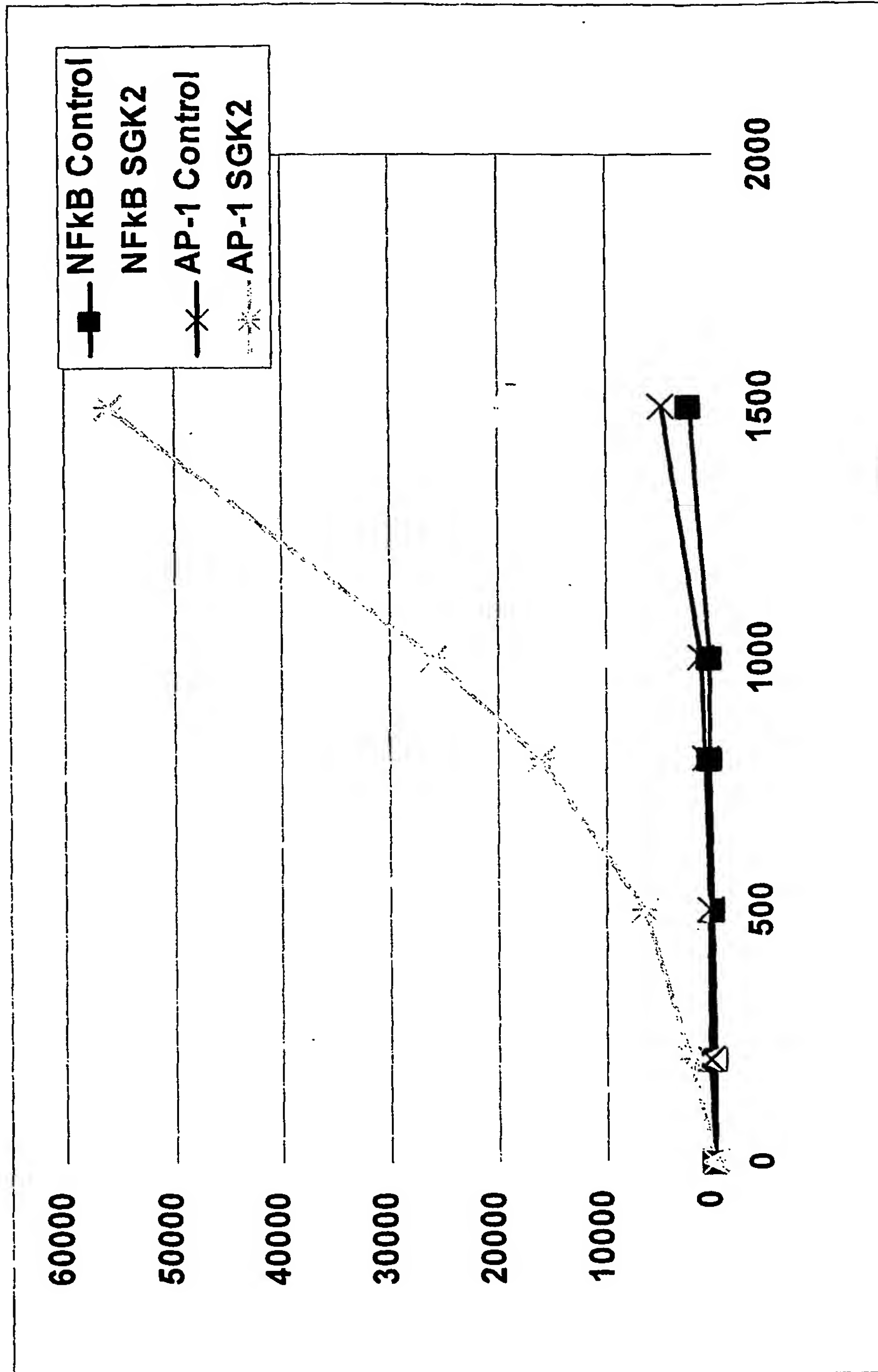


FIGURE 3

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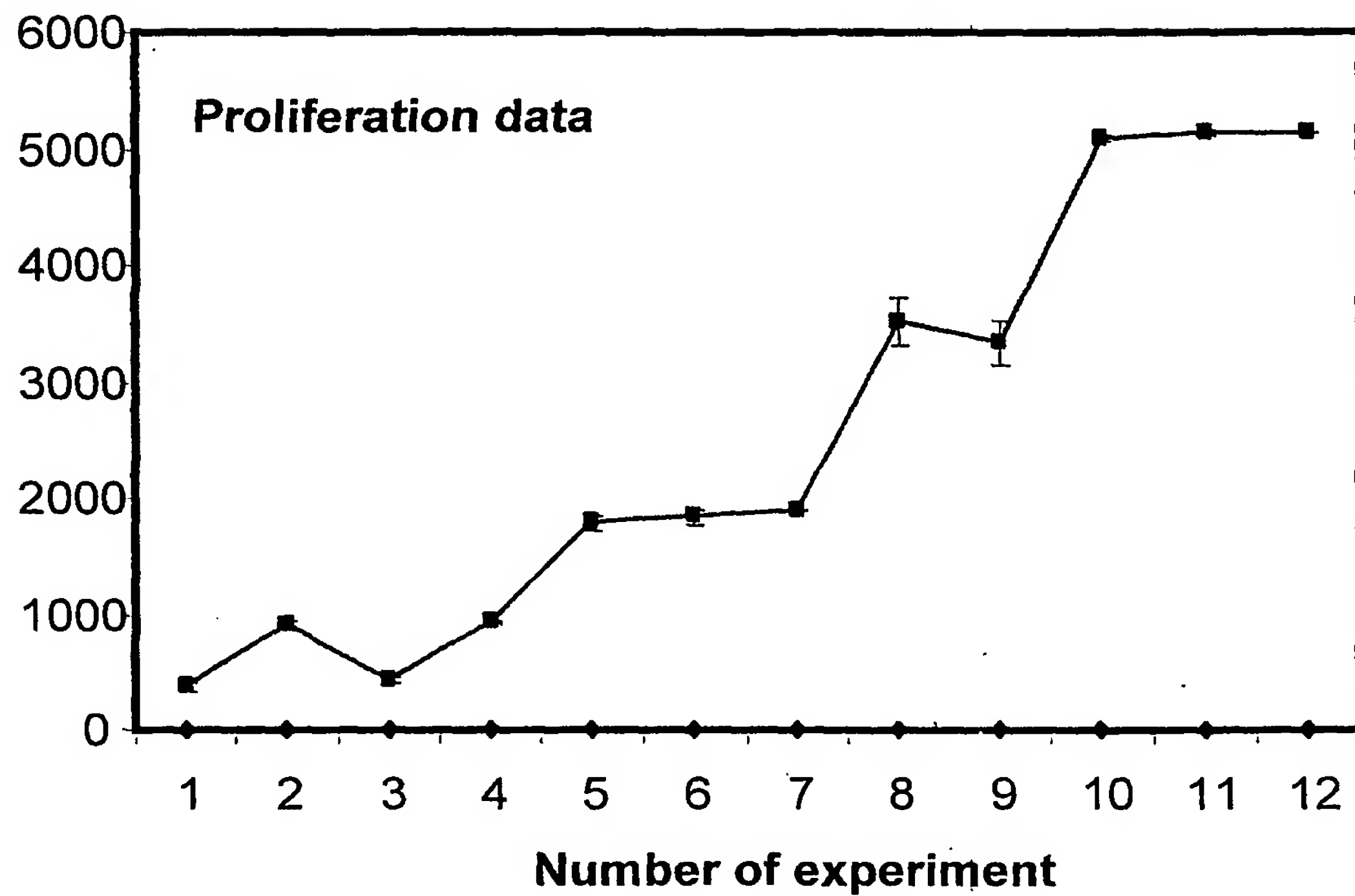
3T3 cells 0.5% Serum

FIGURE 4

- 1) no DNA
- 2) 1 ug/ml pcDNA HIS 3.1
- 3) 0.005 ug/ml K 25/pcDNA HIS 3.1
- 4) 0.1 ug/ml K 25/pcDNA HIS 3.1
- 5) 0.15 ug/ml K 25/pcDNA HIS 3.1
- 6) 0.2 ug/ml K 25/pcDNA HIS 3.1
- 7) 0.25 ug/ml K 25/pcDNA HIS 3.1
- 8) 0.3 ug/ml K 25/pcDNA HIS 3.1
- 9) 0.25 ug/ml K 25/pcDNA HIS 3.1 + 0.1ug/mlPDK 1
- 10) 0.25 ug/ml K 25/pcDNA HIS 3.1 + 0.2ug/mlPDK 1
- 11) 0.375 ug/ml K25/pcDNA HIS 3.1
- 12) 0.5 ug/ml K 25/pcDNA HIS 3.1
- 13) 1 ug/ml K 25/pcDNA HIS 3.1

Alignment of Three DMPK Isoforms Amplified from
Human Brain cDNA Library

WO 02/24947

PCT/IB01/02237

Consensus NM_004409 K216-2 K216-4 K216-5	MSAEVRLRRLLQQLVLDPGFLGLEPLDLLLLGVHQELGASELAQDKYVADFLQWAEPIVVRLKEVRLQRDDFEILKVIGRGAFSEVAVVKMKQTGQVYAMKIMNKWDM MSAEVRLRRLLQQLVLDPGFLGLEPLDLLLLGVHQELGASELAQDKYVADFLQWAEPIVVRLKEVRLQRDDFEILKVIGRGAFSEVAVVKMKQTGQVYAMKIMNKWDM MSAEVRLRRLLQQLVLDPGFLGLEPLDLLLLGVHQELGASELAQDKYVADFLQWAEPIVVRLKEVRLQRDDFEILKVIGRGAFSEVAVVKMKQTGQVYAMKIMNKWDM MSAEVRLRRLLQQLVLDPGFLGLEPLDLLLLGVHQELGASELAQDKYVADFLQWAEPIVVRLKEVRLQRDDFEILKVIGRGAFSEVAVVKMKQTGQVYAMKIMNKWDM MSAEVRLRRLLQQLVLDPGFLGLEPLDLLLLGVHQELGASELAQDKYVADFLQWAEPIVVRLKEVRLQRDDFEILKVIGRGAFSEVAVVKMKQTGQVYAMKIMNKWDM
Consensus NM_004409 K216-2 K216-4 K216-5	LKRGEVSCFREERDVLVNGDRRWITQLHFAFQDENLYLVMEYYVGGDLLTLLSKFGERIPAEMARFYLAIEIVMAIDSVHRLGYVHRDIKPDNILLDRCGHIRLADF LKRGEVSCFREERDVLVNGDRRWITQLHFAFQDENLYLVMEYYVGGDLLTLLSKFGERIPAEMARFYLAIEIVMAIDSVHRLGYVHRDIKPDNILLDRCGHIRLADF LKRGEVSCFREERDVLVNGDRRWITQLHFAFQDENLYLVMEYYVGGDLLTLLSKFGERIPAEMARFYLAIEIVMAIDSVHRLGYVHRDIKPDNILLDRCGHIRLADF LKRGEVSCFREERDVLVNGDRRWITQLHFAFQDENLYLVMEYYVGGDLLTLLSKFGERIPAEMARFYLAIEIVMAIDSVHRLGYVHRDIKPDNILLDRCGHIRLADF LKRGEVSCFREERDVLVNGDRRWITQLHFAFQDENLYLVMEYYVGGDLLTLLSKFGERIPAEMARFYLAIEIVMAIDSVHRLGYVHRDIKPDNILLDRCGHIRLADF
Consensus NM_004409 K216-2 K216-4 K216-5	GSCLKLRADGTVRSLSVAVGTPDYLSPEILQAVGGGPGTSGYGPECDDWALGVFAYEMFYGQTPFYADSTAETYGKIVHYKEHLSLPLVDEGVPEEARDFIQRLLLCPP GSCLKLRADGTVRSLSVAVGTPDYLSPEILQAVGGGPGTSGYGPECDDWALGVFAYEMFYGQTPFYADSTAETYGKIVHYKEHLSLPLVDEGVPEEARDFIQRLLLCPP GSCLKLRADGTVRSLSVAVGTPDYLSPEILQAVGGGPGTSGYGPECDDWALGVFAYEMFYGQTPFYADSTAETYGKIVHYKEHLSLPLVDEGVPEEARDFIQRLLLCPP GSCLKLRADGTVRSLSVAVGTPDYLSPEILQAVGGGPGTSGYGPECDDWALGVFAYEMFYGQTPFYADSTAETYGKIVHYKEHLSLPLVDEGVPEEARDFIQRLSLCPP GSCLKLRADGTVRSLSVAVGTPDYLSPEILQAVGGGPGTSGYGPECDDWALGVFAYEMFYGQTPFYADSTAETYGKIVHYKEHLSLPLVDEGVPEEARDFIQRLLLCPP
Consensus NM_004409 K216-2 K216-4 K216-5	ETRLGRGGAGDFRTHPFFFFGLDWDGLRDSVPPFTPDPEGATDTCNFDLVEDGLTAMVSGGGETLSDIREGAPLGVHLPFVGYSYSCMALRDSEVPGPTPME-EAEQL ETRLGRGGAGDFRTHPFFFFGLDWDGLRDSVPPFTPDPEGATDTCNFDLVEDGLTAMVSGGGETLSDIREGAPLGVHLPFVGYSYSCMALRDSEVPGPTPMEVEAEQL ETRLGRGGAGDFRTHPFFFFGLDWDGLRDSVPPFTPDPEGATDTCNFDLVEDGLTAMVSGGGETLSDIREGAPLGVHLPFVGYSYSCMALRDSEVPGPTPMELEAEQL ETRLGRGGAGDFRTHPFFFFGLDWDGLRDSVPPFTPDPEGATDTCNFDLVEDGLTAM- - -ETLSDIREGAPLGVHLPFVGYSYSCMALRDSEVPGPTPMELEAEQL ETRLGRGGAGDFRTHPFFFFGLDWDGLRDSVPPFTPDPEGATDTCNFDLVEDGLTAMVSGGGETLSDIREGAPLGVHLPFVGYSYSCMALRDSEVPGPTPMEVEAEQL
Consensus NM_004409 K216-2 K216-4 K216-5	LEPHVQAPSLSPQDETAEVAVPAAVPAAEAAEAEVTLRELQEALEEEVLTROSLSREMEAIRTDNQNFASQIREAEARNRDLEAHVRQLQERMELLQAEGATAV LEPHVQAPSLSPQDETAEVAVPAAVPAAEAAEAEVTLRELQEALEEEVLTROSLSREMEAIRTDNQNFASQIREAEARNRDLEAHVRQLQERMELLQAEGATAV LEPHVQAPSLSPQDETAEVAVPAAVPAAEAAEAEVTLRELQEALEEEVLTROSLSREMEAIRTDNQNFASQIREAEARNRDLEAHVRQLQERMELLQAEGATAV LEPHVQAPSLSPQDETAEVAVPAAVPAAEAAEAEVTLRELQEALEEEVLTROSLSREMEAIRTDNQNFASQIREAEARNRDLEAHVRQLQERMELLQAEGATGP LEPHVQAPSLSPQDETAEVAVPAAVPAAEAAEAEVTLRELQEALEEEVLTROSLSREMEAIRTDNQNFASQIREAEARNRDLEAHVRQLQERMELLQAEGATAV
Consensus NM_004409 K216-2 K216-4 K216-5	TGVPSPRATDPPSH- - - - -VPRPGLSEALSLLLFAVLSR-AALGCTGLVAHAGQLTAVWRRPGAARAP TGVPSPRATDPPSHLDGPPAVAVGQCPLVGPGMHRRHLLLPARVPRPGLSEALSLLLFAVLSRAAALGCTGLVAHAGQLTAVWRRPGAARAP TGVPSPRATDPPSHMA-PPAVAVGQCPLVGPGMHRRHLLLPARVPRPGLSEALSLLLFAVLSRSAAALGCTGLVAHAGQLTAVWRRPGAARAP - - - - - TGVPSPRATDPPSH- - - - -VPRPGLSEALSLLLFAVLSRAAALGCTGLVAHAGQLTAVWRRPGAARAP

FIGURE 5

SEQUENCE LISTING

<110> Yoganathan, Thillainathan
Delaney, Allen

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gaggcgctgc ccctgaaggc cccaccttac acttggcggg ggccggagcc aggctcccag 180
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Met Glu Pro Leu Lys Ser Leu Phe Leu Lys Ser Pro Leu Gly Ser Trp
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Asn Gly Ser Gly Ser Gly Gly Gly Gly Gly Gly Gly Gly Gly Arg Pro
20 25 30

gag ggg tct cca aag gca gcg ggt tat gcc aac ccg gtg tgg aca gcc 625
Glu Gly Ser Pro Lys Ala Ala Gly Tyr Ala Asn Pro Val Trp Thr Ala
35 40 45

ctg ttc gac tac gag ccc agt ggg cag gat gag ctg gcc ctg agg aag Leu Phe Asp Tyr Glu Pro Ser Gly Gln Asp Glu Leu Ala Leu Arg Lys 50 55 60	673
ggt gac cgt gtg gag gtg ctg tcc cgg gac gca gcc atc tca gga gac Gly Asp Arg Val Glu Val Leu Ser Arg Asp Ala Ala Ile Ser Gly Asp 65 70 75 80	721
gag ggc tgg tgg gcg ggc cag gtg ggt ggc cag gtg ggc atc ttc ccg Glu Gly Trp Trp Ala Gly Gln Val Gly Gly Gln Val Gly Ile Phe Pro 85 90 95	769
tcc aac tat gtg tct cgg ggt ggc ggc ccg ccc ccc tgc gag gtg gcc Ser Asn Tyr Val Ser Arg Gly Gly Gly Pro Pro Pro Cys Glu Val Ala 100 105 110	817
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ggc aag gtg tac agg ggc agc tgg cga ggt gag ctg gtg gct gtg aag Gly Lys Val Tyr Arg Gly Ser Trp Arg Gly Glu Leu Val Ala Val Lys 130 135 140	913
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Val	Trp	Ser	Phe	Gly	Val	Leu	Leu	Trp	Glu	Leu	Leu	Thr	Gly	Glu	Val	
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cca	tac	cgt	ggc	att	gac	tgc	ctt	gct	gtg	gcc	tat	ggc	gta	gct	gtt	1489
Pro	Tyr	Arg	Gly	Ile	Asp	Cys	Leu	Ala	Val	Ala	Tyr	Gly	Val	Ala	Val	
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aac	aag	ctc	aca	ctg	ccc	atc	cca	tcc	acc	tgc	ccc	gag	ccc	ttc	gca	1537
Asn	Lys	Leu	Thr	Leu	Pro	Ile	Pro	Ser	Thr	Cys	Pro	Glu	Pro	Phe	Ala	
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Gln	Leu	Met	Ala	Asp	Cys	Trp	Ala	Gln	Asp	Pro	His	Arg	Arg	Pro	Asp	
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ttc	gcc	tcc	atc	ctg	cag	cag	ttg	gag	gcg	ctg	gag	gca	cag	gtc	cta	1633
Phe	Ala	Ser	Ile	Leu	Gln	Gln	Leu	Glu	Ala	Leu	Glu	Ala	Gln	Val	Leu	
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cgg	gaa	atg	ccg	cgg	gac	tcc	ttc	cat	tcc	atg	cag	gaa	ggc	tgg	aag	1681
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cgc	gag	atc	cag	ggt	ctc	ttc	gac	gag	ctg	cga	gcc	aag	gaa	aag	gaa	1729
Arg	Glu	Ile	Gln	Gly	Leu	Phe	Asp	Glu	Leu	Arg	Ala	Lys	Glu	Lys	Glu	
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cta	ctg	agc	cgc	gag	gag	gag	ctg	acg	cga	gcg	gcg	cgc	gag	cag	cgg	1777
Leu	Leu	Ser	Arg	Glu	Glu	Glu	Leu	Thr	Arg	Ala	Ala	Arg	Glu	Gln	Arg	
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tca	cag	gcg	gag	cag	ctg	cgg	cgg	cgc	gag	cac	ctg	ctg	gcc	cag	tgg	1825
Ser	Gln	Ala	Glu	Gln	Leu	Arg	Arg	Arg	Glu	His	Leu	Leu	Ala	Gln	Trp	
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Glu	Leu	Glu	Val	Phe	Glu	Arg	Glu	Leu	Thr	Leu	Leu	Leu	Gln	Gln	Val	
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Asp	Arg	Glu	Arg	Pro	His	Val	Arg	Arg	Arg	Arg	Gly	Thr	Phe	Lys	Arg	
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Ser	Lys	Leu	Arg	Ala	Arg	Asp	Gly	Gly	Glu	Arg	Ile	Ser	Met	Pro	Leu	
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Asp	Phe	Lys	His	Arg	Ile	Thr	Val	Gln	Ala	Ser	Pro	Gly	Leu	Asp	Arg	
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ctc aat ggt aac ccc ccg cgg cct agc ctg gag ccc gag gag ccc aag Leu Asn Gly Asn Pro Pro Arg Pro Ser Leu Glu Pro Glu Glu Pro Lys 610 615 620	2353
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Ala	Pro	Trp	Thr	Leu	Phe	Pro	Asp	Ser	Asp	Pro	Phe	Trp	Asp	Ser	Pro	
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Pro	Ala	Asn	Pro	Phe	Gln	Gly	Gly	Pro	Gln	Asp	Cys	Arg	Ala	Gln	Thr	
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Lys	Asp	Met	Gly	Ala	Gln	Ala	Pro	Trp	Val	Pro	Glu	Ala	Gly	Pro		
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Gln	Leu	Met	Ala	Asp	Cys	Trp	Ala	Gln	Asp	Pro	His	Arg	Arg	Pro	Asp
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Ser	Gln	Ala	Glu	Gln	Leu	Arg	Arg	Arg	Glu	His	Leu	Leu	Ala	Gln	Trp
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Arg	Ala	Cys	Trp	Ala	Trp	Gly	Pro	Ser	Ser	Pro	Lys	Pro	Gly	Glu	Ala
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Leu	Gly	Arg	Asp	Leu	Gln	Pro	Pro	Gly	Gly	Pro	Gly	Arg	Glu	Arg	Gly
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Glu	Ser	Pro	Thr	Thr	Pro	Pro	Thr	Pro	Thr	Pro	Ala	Pro	Cys	Pro	Thr
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Glu	Pro	Pro	Pro	Ser	Pro	Leu	Ile	Cys	Phe	Ser	Leu	Lys	Thr	Pro	Asp
	690					695					700				
Ser	Pro	Pro	Thr	Pro	Ala	Pro	Leu	Leu	Leu	Asp	Leu	Gly	Ile	Pro	Val
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Gly	Gln	Arg	Ser	Ala	Lys	Ser	Pro	Arg	Arg	Glu	Glu	Glu	Pro	Arg	Gly
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				Met	Gly	Arg	Lys	Glu	Glu	Asp	Asp	Cys	Ser	Ser	Trp	Lys	Lys	
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Gln	Thr	Thr	Asn	Ile	Arg	Lys	Thr	Phe	Ile	Phe	Met	Glu	Val	Leu	Gly			
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Ser	Gly	Ala	Phe	Ser	Glu	Val	Phe	Leu	Val	Lys	Gln	Arg	Leu	Thr	Gly			
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Lys	Leu	Phe	Ala	Leu	Lys	Cys	Ile	Lys	Lys	Ser	Pro	Ala	Phe	Arg	Asp			
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Ser	Ser	Leu	Glu	Asn	Glu	Ile	Ala	Val	Leu	Lys	Lys	Ile	Lys	His	Glu			
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aac	att	gtg	acc	ctg	gag	gac	atc	tat	gag	agc	acc	acc	cac	tac	tac		351	
Asn	Ile	Val	Thr	Leu	Glu	Asp	Ile	Tyr	Glu	Ser	Thr	Thr	His	Tyr	Tyr			
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ctg	gtc	atg	cag	ctt	ggt	tct	ggt	ggg	gag	ctc	ttt	gac	cgg	atc	ctg		399	
Leu	Val	Met	Gln	Leu	Val	Ser	Gly	Gly	Glu	Leu	Phe	Asp	Arg	Ile	Leu			
95					100					105					110			
gag	cgg	ggt	gtc	tac	aca	gag	aag	gat	gcc	agt	ctg	gtg	atc	cag	cag		447	
Glu	Arg	Gly	Val	Tyr	Thr	Glu	Lys	Asp	Ala	Ser	Leu	Val	Ile	Gln	Gln			
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gtc	ttg	tcg	gca	gtg	aaa	tac	cta	cat	gag	aat	ggc	atc	gtc	cac	aga		495	
Val	Leu	Ser	Ala	Val	Lys	Tyr	Leu	His	Glu	Asn	Gly	Ile	Val	His	Arg			
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Asp Leu Lys Pro Glu Asn Leu Leu Tyr Leu Thr Pro Glu Glu Asn Ser	
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Lys Ile Met Ile Thr Asp Phe Gly Leu Ser Lys Met Glu Gln Asn Gly	
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Ile Met Ser Thr Ala Cys Gly Thr Pro Gly Tyr Val Ala Pro Glu Val	
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ctg gcc cag aaa ccc tac agc aag gct gtg gat tgc tgg tcc atc ggc	687
Leu Ala Gln Lys Pro Tyr Ser Lys Ala Val Asp Cys Trp Ser Ile Gly	
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gtc atc acc tac ata ttg ctc tgt gga tac ccc ccg ttc tat gaa gaa	735
Val Ile Thr Tyr Ile Leu Leu Cys Gly Tyr Pro Pro Phe Tyr Glu Glu	
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Thr Glu Ser Lys Leu Phe Glu Lys Ile Lys Glu Gly Tyr Tyr Glu Phe	
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gag tct cca ttc tgg gat gac att tct gag tca gcc aag gac ttt att	831
Glu Ser Pro Phe Trp Asp Asp Ile Ser Glu Ser Ala Lys Asp Phe Ile	
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tgc cac ttg ctt gag aag gat ccg aac gag cgg tac acc tgt gag aag	879
Cys His Leu Leu Glu Lys Asp Pro Asn Glu Arg Tyr Thr Cys Glu Lys	
255 260 265 270	
gcc ttg agt cat ccc tgg att gac gga aac acg gcc ctc cac cgg gac	927
Ala Leu Ser His Pro Trp Ile Asp Gly Asn Thr Ala Leu His Arg Asp	
275 280 285	
atc tac cca tca gtc agc ctc cag atc cag aag aac ttt gct aag agc	975
Ile Tyr Pro Ser Val Ser Leu Gln Ile Gln Lys Asn Phe Ala Lys Ser	
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Lys Trp Arg Gln Ala Phe Asn Ala Ala Val Val His His Met Arg	
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Lys Leu His Met Asn Leu His Ser Pro Gly Val Arg Pro Glu Val Glu	
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Asn Arg Pro Pro Glu Thr Gln Ala Ser Glu Thr Ser Arg Pro Ser Ser	
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cct gag atc acc atc acc gag gca cct gtc ctg gac cac agt gta gca	1167
Pro Glu Ile Thr Ile Thr Glu Ala Pro Val Leu Asp His Ser Val Ala	
355 360 365	
ctc cct gcc ctg acc caa tta ccc tgc cag cat ggc cgc cgg ccc act	1215
Leu Pro Ala Leu Thr Gln Leu Pro Cys Gln His Gly Arg Arg Pro Thr	
370 375 380	
gcc cct ggt ggc agg tcc ctc aac tgc ctg gtc aat ggc tcc ctc cac	1263
Ala Pro Gly Gly Arg Ser Leu Asn Cys Leu Val Asn Gly Ser Leu His	
385 390 395	

atc agc agc agc ctg gtg ccc atg cat cag ggg tcc ctg gcc gcc ggg 1311
 Ile Ser Ser Ser Leu Val Pro Met His Gln Gly Ser Leu Ala Ala Gly
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ccc tgt ggc tgc tgc tcc agc tgc ctg aac att ggg agc aaa gga aag 1359
 Pro Cys Gly Cys Cys Ser Ser Cys Leu Asn Ile Gly Ser Lys Gly Lys
 415 420 425 430

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 Ser Ser Tyr Cys Ser Glu Pro Thr Leu Leu Lys Lys Ala Asn Lys Lys
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cag aac ttc aag tcg gag gtc atg gta cca gtt aaa gcc agt ggc agc 1455
 Gln Asn Phe Lys Ser Glu Val Met Val Pro Val Lys Ala Ser Gly Ser
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 Ser His Cys Arg Ala Gly Gln Thr Gly Val Cys Leu Ile Met
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<211> 476

<212> PRT

<213> Homo sapiens

<400> 4

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 Phe Ala Leu Lys Cys Ile Lys Lys Ser Pro Ala Phe Arg Asp Ser Ser
 50 55 60
 Leu Glu Asn Glu Ile Ala Val Leu Lys Lys Ile Lys His Glu Asn Ile
 65 70 75 80
 Val Thr Leu Glu Asp Ile Tyr Glu Ser Thr Thr His Tyr Tyr Leu Val
 85 90 95
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 100 105 110
 Gly Val Tyr Thr Glu Lys Asp Ala Ser Leu Val Ile Gln Gln Val Leu
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 130 135 140

Lys Pro Glu Asn Leu Leu Tyr Leu Thr Pro Glu Glu Asn Ser Lys Ile
 145 150 155 160
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 165 170 175
 Ser Thr Ala Cys Gly Thr Pro Gly Tyr Val Ala Pro Glu Val Leu Ala
 180 185 190
 Gln Lys Pro Tyr Ser Lys Ala Val Asp Cys Trp Ser Ile Gly Val Ile
 195 200 205
 Thr Tyr Ile Leu Leu Cys Gly Tyr Pro Pro Phe Tyr Glu Glu Thr Glu
 210 215 220
 Ser Lys Leu Phe Glu Lys Ile Lys Glu Gly Tyr Tyr Glu Phe Glu Ser
 225 230 235 240
 Pro Phe Trp Asp Asp Ile Ser Glu Ser Ala Lys Asp Phe Ile Cys His
 245 250 255
 Leu Leu Glu Lys Asp Pro Asn Glu Arg Tyr Thr Cys Glu Lys Ala Leu
 260 265 270
 Ser His Pro Trp Ile Asp Gly Asn Thr Ala Leu His Arg Asp Ile Tyr
 275 280 285
 Pro Ser Val Ser Leu Gln Ile Gln Lys Asn Phe Ala Lys Ser Lys Trp
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 Arg Gln Ala Phe Asn Ala Ala Val Val His His Met Arg Lys Leu
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 His Met Asn Leu His Ser Pro Gly Val Arg Pro Glu Val Glu Asn Arg
 325 330 335
 Pro Pro Glu Thr Gln Ala Ser Glu Thr Ser Arg Pro Ser Ser Pro Glu
 340 345 350
 Ile Thr Ile Thr Glu Ala Pro Val Leu Asp His Ser Val Ala Leu Pro
 355 360 365
 Ala Leu Thr Gln Leu Pro Cys Gln His Gly Arg Arg Pro Thr Ala Pro
 370 375 380
 Gly Gly Arg Ser Leu Asn Cys Leu Val Asn Gly Ser Leu His Ile Ser
 385 390 395 400
 Ser Ser Leu Val Pro Met His Gln Gly Ser Leu Ala Ala Gly Pro Cys
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 Gly Cys Cys Ser Ser Cys Leu Asn Ile Gly Ser Lys Gly Lys Ser Ser
 420 425 430
 Tyr Cys Ser Glu Pro Thr Leu Leu Lys Lys Ala Asn Lys Lys Gln Asn
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<212> DNA

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<210> 6

<211> 367

<212> PRT

<213> Homo sapiens

<400> 6

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35          40          45
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50          55          60
Val Leu Gln Lys Lys Ser Ile Leu Lys Lys Lys Glu Gln Ser His Ile
65          70          75          80
Met Ala Glu Arg Ser Val Leu Leu Lys Asn Val Arg His Pro Phe Leu
85          90          95
Val Gly Leu Arg Tyr Ser Phe Gln Thr Pro Glu Lys Leu Tyr Phe Val
100         105         110
Leu Asp Tyr Val Asn Gly Gly Glu Leu Phe Phe His Leu Gln Arg Glu
115         120         125
Arg Arg Phe Leu Glu Pro Arg Ala Arg Phe Tyr Ala Ala Glu Val Ala
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Ser Ala Ile Gly Tyr Leu His Ser Leu Asn Ile Ile Tyr Arg Asp Leu
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Lys Pro Glu Asn Ile Leu Leu Asp Cys Gln Gly His Val Val Leu Thr
165         170         175
Asp Phe Gly Leu Cys Lys Glu Gly Val Glu Pro Glu Asp Thr Thr Ser
180         185         190
Thr Phe Cys Gly Thr Pro Glu Tyr Leu Ala Pro Glu Val Leu Arg Lys
195         200         205
Glu Pro Tyr Asp Arg Ala Val Asp Trp Trp Cys Leu Gly Ala Val Leu
210         215         220
Tyr Glu Met Leu His Gly Leu Pro Pro Phe Tyr Ser Gln Asp Val Ser
225         230         235         240
Gln Met Tyr Glu Asn Ile Leu His Gln Pro Leu Gln Ile Pro Gly Gly
245         250         255
Arg Thr Val Ala Ala Cys Asp Leu Leu Gln Ser Leu Leu His Lys Asp
260         265         270
Gln Arg Gln Arg Leu Gly Ser Lys Ala Asp Phe Leu Glu Ile Lys Asn
275         280         285

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His	Val	Phe	Phe	Ser	Pro	Ile	Asn	Trp	Asp	Asp	Leu	Tyr	His	Lys	Arg
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His	Phe	Asp	Pro	Glu	Phe	Thr	Gln	Glu	Ala	Val	Ser	Lys	Ser	Ile	Gly
				325					330					335	
Cys	Thr	Pro	Asp	Thr	Val	Ala	Ser	Ser	Ser	Gly	Ala	Ser	Ser	Ala	Phe
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<210> 7

<211> 2557

<212> DNA

<213> Homo sapiens

<400> 7

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<210> 8
 <211> 590
 <212> PRT
 <213> Homo sapiens

<400> 8

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			20					25					30		
Ile	Leu	Lys	Phe	Pro	His	Ile	Ser	Gln	Cys	Glu	Asp	Leu	Arg	Arg	Thr
		35					40					45			
Ile	Asp	Arg	Asp	Tyr	Cys	Ser	Leu	Cys	Asp	Lys	Gln	Pro	Ile	Gly	Arg
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Leu	Leu	Phe	Arg	Gln	Phe	Cys	Glu	Thr	Arg	Pro	Gly	Leu	Glu	Cys	Tyr
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Ile	Gln	Phe	Leu	Asp	Ser	Val	Ala	Glu	Tyr	Glu	Val	Thr	Pro	Asp	Glu
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Lys	Leu	Gly	Glu	Lys	Gly	Lys	Glu	Ile	Met	Thr	Lys	Tyr	Leu	Thr	Pro
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Lys	Ser	Pro	Val	Phe	Ile	Ala	Gln	Val	Gly	Gln	Asp	Leu	Val	Ser	Gln
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Thr	Glu	Glu	Lys	Leu	Leu	Gln	Lys	Pro	Cys	Lys	Glu	Leu	Phe	Ser	Ala
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Tyr	Leu	Asp	Ser	Met	Phe	Phe	Asp	Arg	Phe	Leu	Gln	Trp	Lys	Trp	Leu
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Glu	Arg	Gln	Pro	Val	Thr	Lys	Asn	Thr	Phe	Arg	Gln	Tyr	Arg	Val	Leu
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Ile	Ala	Thr	Val	Tyr	Gln	Ile	Phe	Pro	Asp	Glu	Val	Leu	Gly	Ser	Gly	
545					550					555					560	
cag	ttt	gga	gtg	gtc	tat	gga	ggg	aaa	cac	cgg	aag	aca	ggc	cgg	gac	1728
Gln	Phe	Gly	Val	Val	Tyr	Gly	Gly	Lys	His	Arg	Lys	Thr	Gly	Arg	Asp	
				565					570					575		
gtg	gca	gtt	aag	gtc	att	gac	aaa	ctg	cgc	ttc	cct	acc	aag	cag	gag	1776
Val	Ala	Val	Lys	Val	Ile	Asp	Lys	Leu	Arg	Phe	Pro	Thr	Lys	Gln	Glu	
			580					585					590			
agc	cag	ctc	cgg	aat	gaa	gtg	gcc	att	ctg	cag	agc	ctg	cgg	cat	ccc	1824
Ser	Gln		Arg	Asn	Glu	Val	Ala	Ile	Leu	Gln	Ser	Leu	Arg	His	Pro	
		595					600					605				
ggg	atc	gtg	aac	ctg	gag	tgc	atg	ttc	gag	acg	cct	gag	aaa	gtg	ttt	1872
Gly	Ile	Val	Asn	Leu	Glu	Cys	Met	Phe	Glu	Thr	Pro	Glu	Lys	Val	Phe	
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gtg	gtg	atg	gag	aag	ctg	cat	ggg	gac	atg	ttg	gag	atg	atc	ctg	tcc	1920
Val	Val	Met	Glu	Lys	Leu	His	Gly	Asp	Met	Leu	Glu	Met	Ile	Leu	Ser	
625					630					635					640	
agt	gag	aag	ggc	cgg	ctg	cct	gag	cgc	ctc	acc	aag	ttc	ctc	atc	acc	1968
Ser	Glu	Lys	Gly	Arg	Leu	Pro	Glu	Arg	Leu	Thr	Lys	Phe	Leu	Ile	Thr	
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cag	atc	ctg	gtg	gct	ttg	aga	cac	ctt	cac	ttc	aag	aac	att	gtc	cac	2016
Gln	Ile	Leu	Val	Ala	Leu	Arg	His	Leu	His	Phe	Lys	Asn	Ile	Val	His	
			660					665					670			
tgt	gac	ttg	aaa	cca	gaa	aac	gtg	ttg	ctg	gca	tca	gca	gac	cca	ttt	2064
Cys	Asp	Leu	Lys	Pro	Glu	Asn	Val	Leu	Leu	Ala	Ser	Ala	Asp	Pro	Phe	
		675					680					685				
cct	cag	gtg	aag	ctg	tgt	gac	ttt	ggc	ttt	gct	cgc	atc	atc	ggc	gag	2112
Pro	Gln	Val	Lys	Leu	Cys	Asp	Phe	Gly	Phe	Ala	Arg	Ile	Ile	Gly	Glu	
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Lys	Ser	Phe	Arg	Arg	Ser	Val	Val	Gly	Thr	Pro	Ala	Tyr	Leu	Ala	Pro	
705					710					715					720	
gag	gtg	ctg	ctc	aac	cag	ggc	tac	aac	cgc	tcg	ctg	gac	atg	tgg	tca	2208
Glu	Val	Leu	Leu	Asn	Gln	Gly	Tyr	Asn	Arg	Ser	Leu	Asp	Met	Trp	Ser	
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gtg	ggc	gtg	atc	atg	tac	gtc	agc	ctc	agc	ggc	acc	ttc	cct	ttc	aac	2256
Val	Gly	Val	Ile	Met	Tyr	Val	Ser	Leu	Ser	Gly	Thr	Phe	Pro	Phe	Asn	
			740					745					750			
gag	gat	gag	gac	atc	aat	gac	cag	atc	cag	aac	gcc	gcc	ttc	atg	tac	2304

Glu	Asp	Glu	Asp	Ile	Asn	Asp	Gln	Ile	Gln	Asn	Ala	Ala	Phe	Met	Tyr		
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ccg	gcc	agc	ccc	tgg	agc	cac	atc	tca	gct	gga	gcc	att	gac	ctc	atc	2352	
Pro	Ala	Ser	Pro	Trp	Ser	His	Ile	Ser	Ala	Gly	Ala	Ile	Asp	Leu	Ile		
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aac	aac	ctg	ctg	cag	gtg	aag	atg	cgc	aaa	cgc	tac	agc	gtg	gac	aaa	2400	
Asn	Asn	Leu	Leu	Gln	Val	Lys	Met	Arg	Lys	Arg	Tyr	Ser	Val	Asp	Lys		
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tct	ctc	agc	cac	ccc	tgg	tta	cag	gag	tac	cag	acg	tgg	ctg	gac	ctc	2448	
Ser	Leu	Ser	His	Pro	Trp	Leu	Gln	Glu	Tyr	Gln	Thr	Trp	Leu	Asp	Leu		
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cga	gag	ctg	gag	ggg	aag	atg	gga	gag	cga	tac	atc	acg	cat	gag	agt	2496	
Arg	Glu	Leu	Glu	Gly	Lys	Met	Gly	Glu	Arg	Tyr	Ile	Thr	His	Glu	Ser		
			820					825					830				
gac	gac	gcg	cgc	tgg	gag	cag	ttt	gca	gca	gag	cat	ccg	ctg	cct	ggg	2544	
Asp	Asp	Ala	Arg	Trp	Glu	Gln	Phe	Ala	Ala	Glu	His	Pro	Leu	Pro	Gly		
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tct	ggg	ctg	ccc	acg	gac	agg	gat	ctc	ggt	ggg	gcc	tgt	cca	cca	cag	2592	
Ser	Gly	Leu	Pro	Thr	Asp	Arg	Asp	Leu	Gly	Gly	Ala	Cys	Pro	Pro	Gln		
	850					855					860						
gac	cac	gac	atg	cag	ggg	ctg	gcg	gag	cgc	atc	agt	gtt	ctc	tga		2637	
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<210> 12
 <211> 878
 <212> PRT
 <213> Homo sapiens

<400> 12

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			20					25					30				
Pro	Leu	Leu	Pro	Gln	Ile	Pro	Ala	Pro	Gly	Ser	Gly	Val	Ser	Phe	His		
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Ile	Gln	Ile	Gly	Leu	Thr	Arg	Glu	Phe	Val	Leu	Leu	Pro	Ala	Ala	Ser		
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Glu	Leu	Ala	His	Val	Lys	Gln	Leu	Ala	Cys	Ser	Ile	Val	Asp	Gln	Lys		
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Phe	Pro	Glu	Cys	Gly	Phe	Tyr	Gly	Leu	Tyr	Asp	Lys	Ile	Leu	Leu	Phe		
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Lys	His	Asp	Pro	Thr	Ser	Ala	Asn	Leu	Leu	Gln	Leu	Val	Arg	Ser	Ser		
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Gly	Asp	Ile	Gln	Glu	Gly	Asp	Leu	Val	Glu	Val	Val	Leu	Ser	Ala	Ser		
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Ala	Thr	Phe	Glu	Asp	Phe	Gln	Ile	Arg	Pro	His	Ala	Leu	Thr	Val	His		
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Ser	Tyr	Arg	Ala	Pro	Ala	Phe	Cys	Asp	His	Cys	Gly	Glu	Met	Leu	Phe		
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Gly	Leu	Val	Arg	Gln	Gly	Leu	Lys	Cys	Asp	Gly	Cys	Gly	Leu	Asn	Tyr		
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His	Lys	Arg	Cys	Ala	Phe	Ser	Ile	Pro	Asn	Asn	Cys	Ser	Gly	Ala	Arg
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Lys	Arg	Arg	Leu	Ser	Ser	Thr	Ser	Leu	Ala	Ser	Gly	His	Ser	Val	Arg
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Leu	Gly	Thr	Ser	Glu	Ser	Leu	Pro	Cys	Thr	Ala	Glu	Glu	Leu	Ser	Arg
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Ser	Thr	Thr	Glu	Leu	Leu	Pro	Arg	Arg	Pro	Pro	Ser	Ser	Ser	Ser	Ser
225				230					235						240
Ser	Ser	Ala	Ser	Ser	Tyr	Thr	Gly	Arg	Pro	Ile	Glu	Leu	Asp	Lys	Met
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Leu	Leu	Ser	Lys	Val	Lys	Val	Pro	His	Thr	Phe	Leu	Ile	His	Ser	Tyr
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Thr	Arg	Pro	Thr	Val	Cys	Gln	Ala	Cys	Lys	Lys	Leu	Leu	Lys	Gly	Leu
		275					280					285			
Phe	Arg	Gln	Gly	Leu	Gln	Cys	Lys	Asp	Cys	Lys	Phe	Asn	Cys	His	Lys
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Arg	Cys	Ala	Thr	Arg	Val	Pro	Asn	Asp	Cys	Leu	Gly	Glu	Ala	Leu	Ile
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Asn	Gly	Asp	Val	Pro	Met	Glu	Glu	Ala	Thr	Asp	Phe	Ser	Glu	Ala	Asp
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Lys	Ser	Ala	Leu	Met	Asp	Glu	Ser	Glu	Asp	Ser	Gly	Val	Ile	Pro	Gly
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Ser	His	Ser	Glu	Asn	Ala	Leu	His	Ala	Ser	Glu	Glu	Glu	Glu	Gly	Glu
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Gly	Gly	Lys	Ala	Gln	Ser	Ser	Leu	Gly	Tyr	Ile	Pro	Leu	Met	Arg	Val
	370					375					380				
Val	Gln	Ser	Val	Arg	His	Thr	Thr	Arg	Lys	Ser	Ser	Thr	Thr	Leu	Arg
385				390						395					400
Glu	Gly	Trp	Val	Val	His	Tyr	Ser	Asn	Lys	Asp	Thr	Leu	Arg	Lys	Arg
			405						410					415	
His	Tyr	Trp	Arg	Leu	Asp	Cys	Lys	Cys	Ile	Thr	Leu	Phe	Gln	Asn	Asn
			420					425					430		
Thr	Thr	Asn	Arg	Tyr	Tyr	Lys	Glu	Ile	Pro	Leu	Ser	Glu	Ile	Leu	Thr
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Val	Glu	Ser	Ala	Gln	Asn	Phe	Ser	Leu	Val	Pro	Pro	Gly	Thr	Asn	Pro
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His	Cys	Phe	Glu	Ile	Val	Thr	Ala	Asn	Ala	Thr	Tyr	Phe	Val	Gly	Glu
465				470						475					480
Met	Pro	Gly	Gly	Thr	Pro	Gly	Gly	Pro	Ser	Gly	Gln	Gly	Ala	Glu	Ala
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Ala	Arg	Gly	Trp	Glu	Thr	Ala	Ile	Arg	Gln	Ala	Leu	Met	Pro	Val	Ile
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Leu	Gln	Asp	Ala	Pro	Ser	Ala	Pro	Gly	His	Ala	Pro	His	Arg	Gln	Ala
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Gln	Phe	Gly	Val	Val	Tyr	Gly	Gly	Lys	His	Arg	Lys	Thr	Gly	Arg	Asp
			565					570						575	
Val	Ala	Val	Lys	Val	Ile	Asp	Lys	Leu	Arg	Phe	Pro	Thr	Lys	Gln	Glu
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Ser	Gln	Leu	Arg	Asn	Glu	Val	Ala	Ile	Leu	Gln	Ser	Leu	Arg	His	Pro
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Gly	Ile	Val	Asn	Leu	Glu	Cys	Met	Phe	Glu	Thr	Pro	Glu	Lys	Val	Phe
	610					615					620				
Val	Val	Met	Glu	Lys	Leu	His	Gly	Asp	Met	Leu	Glu	Met	Ile	Leu	Ser
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Ser	Glu	Lys	Gly	Arg	Leu	Pro	Glu	Arg	Leu	Thr	Lys	Phe	Leu	Ile	Thr
			645					650						655	
Gln	Ile	Leu	Val	Ala	Leu	Arg	His	Leu	His	Phe	Lys	Asn	Ile	Val	His
			660					665					670		

Cys	Asp	Leu	Lys	Pro	Glu	Asn	Val	Leu	Leu	Ala	Ser	Ala	Asp	Pro	Phe
		675					680					685			
Pro	Gln	Val	Lys	Leu	Cys	Asp	Phe	Gly	Phe	Ala	Arg	Ile	Ile	Gly	Glu
		690				695					700				
Lys	Ser	Phe	Arg	Arg	Ser	Val	Val	Gly	Thr	Pro	Ala	Tyr	Leu	Ala	Pro
		705			710					715					720
Glu	Val	Leu	Leu	Asn	Gln	Gly	Tyr	Asn	Arg	Ser	Leu	Asp	Met	Trp	Ser
				725				730						735	
Val	Gly	Val	Ile	Met	Tyr	Val	Ser	Leu	Ser	Gly	Thr	Phe	Pro	Phe	Asn
			740					745					750		
Glu	Asp	Glu	Asp	Ile	Asn	Asp	Gln	Ile	Gln	Asn	Ala	Ala	Phe	Met	Tyr
		755					760					765			
Pro	Ala	Ser	Pro	Trp	Ser	His	Ile	Ser	Ala	Gly	Ala	Ile	Asp	Leu	Ile
		770				775					780				
Asn	Asn	Leu	Leu	Gln	Val	Lys	Met	Arg	Lys	Arg	Tyr	Ser	Val	Asp	Lys
				790						795					800
Ser	Leu	Ser	His	Pro	Trp	Leu	Gln	Glu	Tyr	Gln	Thr	Trp	Leu	Asp	Leu
				805				810						815	
Arg	Glu	Leu	Glu	Gly	Lys	Met	Gly	Glu	Arg	Tyr	Ile	Thr	His	Glu	Ser
			820					825					830		
Asp	Asp	Ala	Arg	Trp	Glu	Gln	Phe	Ala	Ala	Glu	His	Pro	Leu	Pro	Gly
		835					840					845			
Ser	Gly	Leu	Pro	Thr	Asp	Arg	Asp	Leu	Gly	Gly	Ala	Cys	Pro	Pro	Gln
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<211> 2037

<212> DNA

<213> Homo sapiens

<220>

<221> CDS

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ccg	ggg	tct	cct	ccg	ccc	ccc	ggc	ggc	cta	gag	ctg	cag	tcg	ccg	cca	96
Pro	Gly	Ser	Pro	Pro	Pro	Pro	Gly	Gly	Leu	Glu	Leu	Gln	Ser	Pro	Pro	
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ccg	cta	ctg	ccc	cag	atc	ccg	gcc	ccg	ggt	tcc	ggg	gtc	tcc	ttt	cac	144
Pro	Leu	Leu	Pro	Gln	Ile	Pro	Ala	Pro	Gly	Ser	Gly	Val	Ser	Phe	His	
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atc	cag	atc	ggg	ctg	acc	cgc	gag	ttc	gtg	ctg	ttg	ccc	gcc	gcc	tcc	192
Ile	Gln	Ile	Gly	Leu	Thr	Arg	Glu	Phe	Val	Leu	Leu	Pro	Ala	Ala	Ser	
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gag	ctg	gct	cat	gtg	aag	cag	ctg	gcc	tgt	tcc	atc	gtg	gac	cag	aag	240
Glu	Leu	Ala	His	Val	Lys	Gln	Leu	Ala	Cys	Ser	Ile	Val	Asp	Gln	Lys	
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ttc	cct	gag	tgt	ggc	ttc	tac	ggc	ctt	tac	gac	aag	atc	ctg	ctt	ttc	288
Phe	Pro	Glu	Cys	Gly	Phe	Tyr	Gly	Leu	Tyr	Asp	Lys	Ile	Leu	Leu	Phe	
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Lys His Asp Pro Thr Ser Ala Asn Leu Leu Gln Leu Val Arg Ser Ser	
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gga gac atc cag gag ggc gac ctg gtg gag gtg gtg ctg tcg gcc tcg	384
Gly Asp Ile Gln Glu Gly Asp Leu Val Glu Val Val Leu Ser Ala Ser	
115 120 125	
gcc acc ttc gag gac ttc cag atc cgc ccg cac gcc ctc acg gtg cac	432
Ala Thr Phe Glu Asp Phe Gln Ile Arg Pro His Ala Leu Thr Val His	
130 135 140	
tcc tat cgg gcg cct gcc ttc tgt gat cac tgc ggg gag atg ctc ttc	480
Ser Tyr Arg Ala Pro Ala Phe Cys Asp His Cys Gly Glu Met Leu Phe	
145 150 155 160	
ggc cta gtg cgc cag ggc ctc aag tgc gat ggc tgc ggg ctg aac tac	528
Gly Leu Val Arg Gln Gly Leu Lys Cys Asp Gly Cys Gly Leu Asn Tyr	
165 170 175	
cac aag cgc tgt gcc ttc agc atc ccc aac aac tgt agt ggg gcc cgc	576
His Lys Arg Cys Ala Phe Ser Ile Pro Asn Asn Cys Ser Gly Ala Arg	
180 185 190	
aaa cgg cgc ctg tca tcc acg tct ctg gcc agt ggc cac tcg gtg cgc	624
Lys Arg Arg Leu Ser Ser Thr Ser Leu Ala Ser Gly His Ser Val Arg	
195 200 205	
ctc ggc acc tcc gag tcc ctg ccc tgc acg gct gaa gag ctg agc cgt	672
Leu Gly Thr Ser Glu Ser Leu Pro Cys Thr Ala Glu Glu Leu Ser Arg	
210 215 220	
agc acc acc gaa ctc ctg cct cgc cgt ccc ccg tca tcc tct tcc tcc	720
Ser Thr Thr Glu Leu Leu Pro Arg Arg Pro Pro Ser Ser Ser Ser	
225 230 235 240	
tct tct gcc tca tcg tat acg ggc cgc ccc att gag ctg gac aag atg	768
Ser Ser Ala Ser Ser Tyr Thr Gly Arg Pro Ile Glu Leu Asp Lys Met	
245 250 255	
ctg ctc tcc aag gtc aag gtg ccg cac acc ttc ctc atc cac agc tat	816
Leu Leu Ser Lys Val Lys Val Pro His Thr Phe Leu Ile His Ser Tyr	
260 265 270	
aca cgg ccc acc gtt tgc cag gct tgc aag aaa ctc ctc aag ggc ctc	864
Thr Arg Pro Thr Val Cys Gln Ala Cys Lys Lys Leu Leu Lys Gly Leu	
275 280 285	
ttc cgg cag ggc ctg caa tgc aaa gac tgc aag ttt aac tgt cac aaa	912
Phe Arg Gln Gly Leu Gln Cys Lys Asp Cys Lys Phe Asn Cys His Lys	
290 295 300	
cgc tgc gcc acc cgc gtc cct aat gac tgc ctg ggg gag gcc ctt atc	960
Arg Cys Ala Thr Arg Val Pro Asn Asp Cys Leu Gly Glu Ala Leu Ile	
305 310 315 320	
aat gga gat gtg ccg atg gag gag gcc acc gat ttc agc gag gct gac	1008
Asn Gly Asp Val Pro Met Glu Glu Ala Thr Asp Phe Ser Glu Ala Asp	
325 330 335	
aag agc gcc ctc atg gat gag tca gag gac tcc ggt gtc atc cct ggc	1056

Lys	Ser	Ala	Leu	Met	Asp	Glu	Ser	Glu	Asp	Ser	Gly	Val	Ile	Pro	Gly	
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Ser	His	Ser	Glu	Asn	Ala	Leu	His	Ala	Ser	Glu	Glu	Glu	Glu	Gly	Glu	
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Gly	Gly	Lys	Ala	Gln	Ser	Ser	Leu	Gly	Tyr	Ile	Pro	Leu	Met	Arg	Val	
	370					375					380					
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Val	Gln	Ser	Val	Arg	His	Thr	Thr	Arg	Lys	Ser	Ser	Thr	Thr	Leu	Arg	
385					390					395					400	
gag	ggt	tgg	gtg	gtt	cat	tac	agc	aac	aag	gac	acg	ctg	aga	aag	cgg	1248
Glu	Gly	Trp	Val	Val	His	Tyr	Ser	Asn	Lys	Asp	Thr	Leu	Arg	Lys	Arg	
				405				410						415		
cac	tat	tgg	cgc	ctg	gac	tgc	aag	tgt	atc	acg	ctc	ttc	cag	aac	aac	1296
His	Tyr	Trp	Arg	Leu	Asp	Cys	Lys	Cys	Ile	Thr	Leu	Phe	Gln	Asn	Asn	
			420					425					430			
acg	acc	aac	aga	tac	tat	aag	gaa	att	ccg	ctg	tca	gaa	atc	ctc	acg	1344
Thr	Thr	Asn	Arg	Tyr	Tyr	Lys	Glu	Ile	Pro	Leu	Ser	Glu	Ile	Leu	Thr	
		435					440					445				
gtg	gag	tcc	gcc	cag	aac	ttc	agc	ctt	gtg	ccg	ccg	ggc	acc	aac	cca	1392
Val	Glu	Ser	Ala	Gln	Asn	Phe	Ser	Leu	Val	Pro	Pro	Gly	Thr	Asn	Pro	
	450					455					460					
cac	tgc	ttt	gag	atc	gtc	act	gcc	aat	gcc	acc	tac	ttc	gtg	ggc	gag	1440
His	Cys	Phe	Glu	Ile	Val	Thr	Ala	Asn	Ala	Thr	Tyr	Phe	Val	Gly	Glu	
465					470				475						480	
atg	cct	ggc	ggg	act	ccg	ggt	ggg	cca	agt	ggg	cag	ggg	gct	gag	gcc	1488
Met	Pro	Gly	Gly	Thr	Pro	Gly	Gly	Pro	Ser	Gly	Gln	Gly	Ala	Glu	Ala	
				485				490						495		
gcc	cgg	ggc	tgg	gag	aca	gcc	atc	cgc	cag	gcc	ctg	atg	ccc	gtc	atc	1536
Ala	Arg	Gly	Trp	Glu	Thr	Ala	Ile	Arg	Gln	Ala	Leu	Met	Pro	Val	Ile	
			500					505					510			
ctt	cag	gac	gca	ccc	agc	gcc	cca	ggc	cac	gcg	ccc	cac	aga	caa	gct	1584
Leu	Gln	Asp	Ala	Pro	Ser	Ala	Pro	Gly	His	Ala	Pro	His	Arg	Gln	Ala	
		515					520					525				
tct	ctg	agc	atc	tct	gtg	tcc	aac	agt	cag	atc	caa	gag	aat	gtg	gac	1632
Ser	Leu	Ser	Ile	Ser	Val	Ser	Asn	Ser	Gln	Ile	Gln	Glu	Asn	Val	Asp	
	530					535					540					
att	gcc	act	gtc	tac	cag	atc	ttc	cct	gac	gaa	gtg	ctg	ggc	tca	ggg	1680
Ile	Ala	Thr	Val	Tyr	Gln	Ile	Phe	Pro	Asp	Glu	Val	Leu	Gly	Ser	Gly	
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Pro	Glu	Cys	Asp	Trp	Trp	Ala	Leu	Gly	Val	Phe	Ala	Tyr	Glu	Met	Phe	260	265	270
Tyr	Gly	Gln	Thr	Pro	Phe	Tyr	Ala	Asp	Ser	Thr	Ala	Glu	Thr	Tyr	Gly	275	280	285
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Pro	Glu	Thr	Arg	Leu	Gly	Arg	Gly	Gly	Ala	Gly	Asp	Phe	Arg	Thr	His	325	330	335
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(57) Abstract: Detection of expression of the provided protein kinase in cancers is useful as a diagnostic, for determining the effectiveness of drugs, and determining patient prognosis. The encoded polypeptides further provides a target for screening pharmaceutical agents effective in inhibiting the growth or metastasis of tumor cells.

INTERNATIONAL SEARCH REPORT

In Application No
PCT/IB 01/02237

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12Q1/68 G01N33/574 A61P35/00 A61K31/70 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N C12Q C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, MEDLINE, EMBASE, SCISEARCH, CHEM ABS Data, SEQUENCE SEARCH, BIOSIS, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 65928 A (SHANKARA SRINIVAS ; GENZYME CORP (US); ROBERTS BRUCE L (US)) 23 December 1999 (1999-12-23) page 100, line 33; claims 1,9-11 ---	1-16
X	ING Y L ET AL: "MLK-3: IDENTIFICATION OF A WIDELY-EXPRESSED PROTEIN KINASE BEARING AN SH3 DOMAIN AND A LEUCINE ZIPPER-BASIC REGION DOMAIN" ONCOGENE, BASINGSTOKE, HANTS, GB, vol. 9, no. 6, June 1994 (1994-06), pages 1745-1750, XP001087926 ISSN: 0950-9232 cited in the application MLK-3 = SEQ ID NO:1 --- -/--	16



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "8" document member of the same patent family

Date of the actual completion of the international search

18 February 2003

Date of mailing of the international search report

05. 03. 2003

Name and mailing address of the ISA

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Gabriels, J

INTERNATIONAL SEARCH REPORT

Int'l Application No
PCT/IB 01/02237

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *

Citation of document, with indication, where appropriate, of the relevant passages

Relevant to claim No.

A

WO 99 53036 A (SUGEN INC ;WHYTE DAVID
(US); PLOWMAN GREGORY (US); MARTINEZ
RICARD) 21 October 1999 (1999-10-21).
page 134, line 25 -page 137, line 19;
claims 23,24

1-15

A

GIOELI DANIEL ET AL: "Activation of
mitogen-activated protein kinase
associated with prostate cancer
progression."
CANCER RESEARCH,
vol. 59, no. 2,
15 January 1999 (1999-01-15), pages
279-284, XP002222312
ISSN: 0008-5472
page 280 -page 281

1-15

A

SIVARAMAN V S ET AL: "HYPEREXPRESSION OF
MITOGEN-ACTIVATED PROTEIN KINASE IN HUMEN
BREASTCANCER"
JOURNAL OF CLINICAL INVESTIGATION, NEW
YORK, NY, US,
vol. 99, 1 April 1997 (1997-04-01), pages
1478-1483, XP002070096
ISSN: 0021-9738
figures 1,2

1-15

X

DATABASE EMBL 'Online!
EBI; 21 April 1999 (1999-04-21)
RHODES S:
retrieved from EMBL, accession no.
HS272L161
Database accession no. AL049688
XP002231579
a part of SEQ ID NO:3 is identical to
AL049688 (99,9%) and the protein sequence
encoded by AL049688 (Q9Y3J7) is identical
to SEQ ID NO:4 of the present
application.
abstract

16

X

WO 00 35946 A (UNIV DUNDEE ;COHEN PHILIP
(GB); DEAK MARIA (GB); KOBAYASHI TAKAYAS)
22 June 2000 (2000-06-22)
SGK2-alpha (SEQ 7 from Patent W00035946) =
SEQ ID NO:5
claims 25-33

1-16

INTERNATIONAL SEARCH REPORT

Int. Application No
PCT/IB 01/02237

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		Relevant to claim No.
Category *	Citation of document, with indication, where appropriate, of the relevant passages	
P, X	WO 00 73469 A (PLOWMAN GREGORY D ; SUGEN INC (US); WHYTE DAVID (US); MARTINEZ RICA) 7 December 2000 (2000-12-07) SEQ ID NO:118 from Patent W00073469 = SEQ ID NO:5 (also describes SEQ ID NO:6 = protein) SEQ ID NO: 130 from Patent W00073469 = is part of in SEQ ID NO: 14 claims 1,33 example 3	1-16
P, X	WO 01 44497 A (UNIV DUNDEE ; ALESSI DARIO (GB); BIONDI RICARDO (GB)) 21 June 2001 (2001-06-21) SGK2 (fig 16) = 317 AA of SEQ ID NO:6 claim 1	1 16
X	KUNAPULI PRIYA ET AL: "Cloning and expression of GRK5: A member of the G protein-coupled receptor kinase family." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 90, no. 12, 1993, pages 5588-5592, XP002231578 1993 ISSN: 0027-8424 SEQ ID NO 7 = GRK5 SEQ ID NO 8 = GRK5 protein figure 2	1-16
X	WO 98 53050 A (BEN SASSON SHMUEL A ; YISSUM RES DEV CO (IL); CHILDRENS MEDICAL CEN) 26 November 1998 (1998-11-26) SEQ ID NO 7 = GRK5 SEQ ID NO 8 = GRK5 protein figure 3C	1
X	WO 00 18895 A (YISSUM RES DEV CO ; CHILDRENS MEDICAL CENTER (US)) 6 April 2000 (2000-04-06) SEQ ID NO:21 from Patent W09316196 (alpha D peptide) is a part of SEQ ID NO:8 of the present application. claims 61-67	16
X	WO 93 16196 A (KORNELUK ROBERT G ; MAHADEVAN MANI S (CA)) 19 August 1993 (1993-08-19) AAQ53993 (EMBL) = SEQ ID NO:9 AAR38860 (EMBL) = SEQ ID NO:10 (isoform SEQ ID NO:39) page 41 -page 47	

INTERNATIONAL SEARCH REPORT

Inte Application No

PCT/IB 01/02237

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 01 81555 A (INCYTE GENOMICS INC) 1 November 2001 (2001-11-01) SEQ ID NO:23 from WO0181555 = SEQ ID NO:11 (99,9%) SEQ ID NO:5 from WO0181555 = SEQ ID NO:12 claims 1,5 ---	1-16
E	WO 02 22795 A (PE CORP NY) 21 March 2002 (2002-03-21) AAD36021 (EMBL) from WO0222795 = SEQ ID NO:11 (99,9%) SEQ ID NO:2 from WO0222795 = SEQ ID NO:12 claims 1,4 -----	1-16

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB 01/02237

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box 1.2

Present claim 9 relates to a method using compounds defined by reference to a desirable characteristic or property, namely being an inhibitor of kinase activity.

The claim covers all compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the product compound by reference to a result as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those compounds which appear to be clear, supported and disclosed, namely those parts relating to antisense sequences of SEQ ID NO:1,3,5,7,9,11, and 13 as inhibitor of kinase activity.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

Invention 1: claims 1-16 (all partially)

An isolated nucleic acid comprising the sequence of SEQ ID NO:1 and methods using this sequence or the polypeptide encoded thereby (SEQ ID NO:2) for screening for anticancer agents, diagnosing cancer, and inhibiting the growth of a cancer cell.

Invention 2: claims 1-16 (all partially)

An isolated nucleic acid comprising the sequence of SEQ ID NO:3 and methods using this sequence or the polypeptide encoded thereby (SEQ ID NO:4) for screening for anticancer agents, diagnosing cancer, and inhibiting the growth of a cancer cell.

Invention 3: claims 1-16 (all partially)

An isolated nucleic acid comprising the sequence of SEQ ID NO:5 and methods using this sequence or the polypeptide encoded thereby (SEQ ID NO:6) for screening for anticancer agents, diagnosing cancer, and inhibiting the growth of a cancer cell.

Invention 4: claims 1-16 (all partially)

An isolated nucleic acid comprising the sequence of SEQ ID NO:7 and methods using this sequence or the polypeptide encoded thereby (SEQ ID NO:8) for screening for anticancer agents, diagnosing cancer, and inhibiting the growth of a cancer cell.

Invention 5: claims 1-16 (all partially)

An isolated nucleic acid comprising the sequence of SEQ ID NO:9 and methods using this sequence or the polypeptide encoded thereby (SEQ ID NO:10) or its isoforms (SEQ ID NO:38 and 39) for screening for anticancer agents, diagnosing cancer, and inhibiting the growth of a cancer cell.

Invention 6: claims 1-16 (all partially)

An isolated nucleic acid comprising the sequence of SEQ ID NO:11 and methods using this sequence or the polypeptide encoded thereby (SEQ ID NO:12) for screening for anticancer agents, diagnosing cancer, and inhibiting the growth of a cancer cell.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Invention 7: claims 1-16 (all partially)
An isolated nucleic acid comprising the sequence of SEQ ID NO:13 and methods using this sequence or the polypeptide encoded thereby (SEQ ID NO:14) for screening for anticancer agents, diagnosing cancer, and inhibiting the growth of a cancer cell.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter al Application No

PCT/IB 01/02237

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Form PCT/ISA/210 (patent family annex) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IB 01/02237

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